THE ROLE OF POLO-LIKE KINASE 4 (PLK4) IN CHROMOSOME SEGREGATION AND GENOME MAINTENANCE IN MAMMALIAN MEIOSIS

by

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ABSTRACT

Polo-like kinase 4 (PLK4) belongs to the family of polo-like kinases (PLK1-5), an evolutionarily conserved family of serine/threonine kinases containing a characteristic polo box and similar architecture. PLK4 differs from the other polo-like kinases by possessing a structurally divergent sequence and therefore different substrate specificity and mechanism of action. Previous studies of PLK4 have centered on its role in centriole biogenesis. Overexpression of PLK4 has been shown to cause centrosome amplification and supernumerary centrosomes are a signature event in tumorigenesis and cancer. Recently, PLK4 has been implicated in the process of spermatogenesis. Mutation within the kinase domain of PLK4 was found to cause hypogonadism and germ cell loss. Our lab also reported that PLK4 localizes to the largely unsynapsed X and Y axes during meiotic prophase I, displaying a similar pattern to proteins involved in the process of meiotic sex chromosome inactivation (MSCI). These findings suggests that PLK4 harbors a novel role in MSCI during spermatogenesis and aberrancies in PLK4 function leads to meiotic arrest, loss of germ cells and infertility.

Using the potent and reversible small molecule inhibitor of PLK4, centrinone as well as mutant mice bearing a mutation in the kinase domain of PLK4, we assess the progression of meiosis to check for defects specifically at prophase I stage while comparing the observations to their wild type littermates. Our data exhibits delays in meiotic progression as well as an indicating misregulation of DNA damage response in centrinone treated cells.

Based on existing literature as well as findings from this study, we present strong evidence that the protein PLK4 has a role to play in accurate chromosome segregation and MSCI during prophase I of male meiosis. Inhibition or mutation of PLK4 results in meiotic failure which eventually leads to apoptosis and loss of germ cells. The mechanistic details and full repertoire of proteins and their substrates involved in this process is still being studied and we propose PLK4 as a key player in MSCI and spermatogenesis.

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Finally, I must express my profound gratitude to my family for their continuous encouragement and for being my support system and most importantly my gratitude goes to the Almighty God for His grace that has brought me this far.

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INTRODUCTION

OVERVIEW OF MEIOTIC PROPHASE I

Meiosis is an indispensable event in the formation of gametes in all sexual reproducing species, it involves one round of chromosome replication followed by two subsequent rounds of chromosome segregation - a reductive segregation in which homologous chromosomes separate from one another (meiosis I) and an equational segregation in which sister chromatids separate from each other (meiosis II) to yield four haploid gametes during spermatogenesis (**Figure 1)**. During the extended meiotic prophase I, homologous chromosomes recognize, pair and synapse with one another and undergo crossover recombination, a process mediated by the unique meiotic scaffold called the synaptonemal complex (SC). Meiotic recombination generates diversity within a population and also creates the connection between homologous chromosomes - the chiasmata that holds the chromosomes in opposition on the meiotic spindle and ensure their accurate segregation. The substages of meiosis I prophase are defined by chromosome configurations and structure: During leptonema (leptotene stage), the chromatids experience genetically programmed double-strand DNA breaks induced by the meiotic specific topoisomerase II-like enzyme, sporulation 11 (SPO 11) and this provides the substrate for recombination, the homologous chromosomes begin to align at this stage but are not yet paired. By zygonema (zygotene stage), homologous chromosomes have found each other, pairing extends and synapsis is initiated. Pachynema (pachytene stage) is defined by completion of synapsis and

compartmentalization of the X and Y chromosomes into a nuclear subdomain known as the sex body. Chromosomes undergo desynapsis in the final diplotene stage (diplonema). Summarily, pairing occurs during leptotene and zygotene stages, autosomal synapsis is completed at pachytene stage and desynapsis occurs during diplotene stage (**Figure 2).** Correct execution of this process is essential for maintaining the integrity of the genome, for ensuring normal development of offspring and for fertility and so must be accurately regulated (Handel, 2004; Handel and Schimenti, 2010). The regulation and control of cell division processes has been established to rely on the complex interplay between kinases and phosphatases which are conserved among species. Eukaryotic protein kinases constitute one of the largest of mammalian gene families, the mouse genome for example encodes 540 protein kinases. These kinases, by adding phosphate groups to their substrates alter the activity, location and lifetime of a large fraction of proteins and coordinate several complex cellular functions (Caenepeel et al., 2004). Key kinases known to be involved in the cell cycle progression include a series of conserved serine/threonine protein kinases such as the cyclin-dependent kinases, aurora kinases and polo-like kinases (Barr et al., 2011).

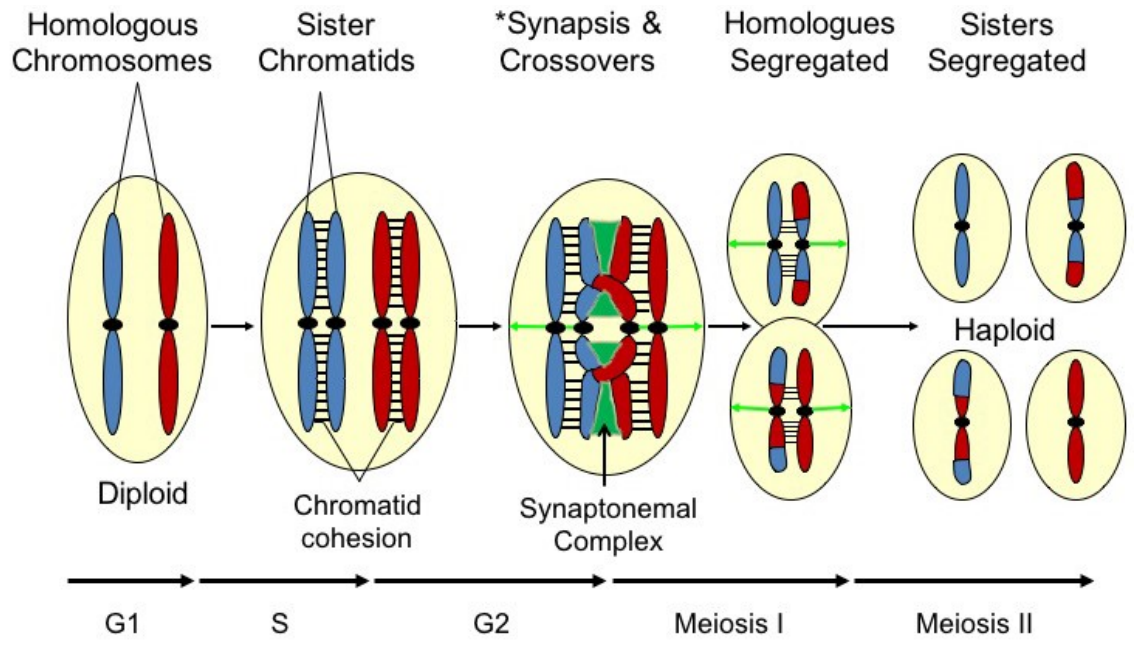


Figure 1: An overview of meiosis. Figure created by Dr. Phil Jordan

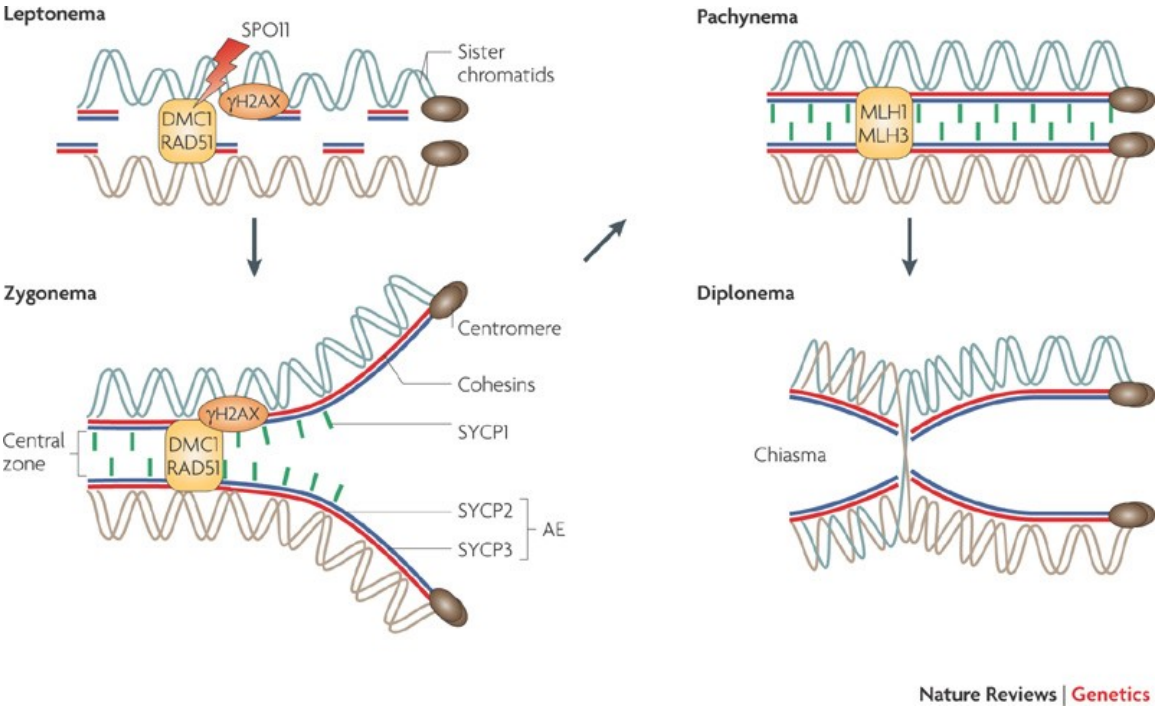


Figure 2: The substages of meiosis I prophase in mammals (Handel and Schimenti, 2010)

POLO-LIKE KINASES – STRUCTURE AND DOMAINS

Polo-like kinases are potent regulators of the cell cycle and are conserved from yeasts to humans (Archambault and Glover, 2009). They were first discovered in *Drosophila melanogaster.* Mutation of *polo* caused abnormal centrosomes and monopolar spindles (Sunkel and Glover, 1988). Single polo-like kinases were also found to be present in budding yeast (*Saccharomyces cerevisiae)* and fission yeast (*Schizosaccharomyces pombe*) as cell cycle division 5 (Cdc5) and Plo1 respectively, and they function to regulate mitotic entry, exit and cytokinesis. Research on this family of kinases after their discovery in budding yeast (Cdc5), *Drosophila melanogaster* (Polo) and mammals (polo-like kinases) have shown that they are present in all branches of the Eukarya and there are five paralogs in mammals- PLK1-5 (Carvalho-santos et al, 2010). The polo-like kinase family are serine/threonine kinases, all possessing a characteristic polo box and sharing similar architecture: an amino-terminal (Serine/Threonine) catalytic kinase domain and a carboxy-terminal region containing two or more polo boxes. (Kothe, M et al., 2007). Among the polo-like kinases, PLK1-3 share very similar catalytic domains with two polo-box domains at their C-terminus (**Figure 3**). PLK5 does not seem to have a role in cell cycle progression, and does not possess a functional kinase domain. However, it is detected in the cytoplasm of neurons and glia and possess nervous system specific functions and tumor suppressor activity in the brain and cancer (de Carcer et al, 2011). PLK4, sometimes referred to as SAK (**S**nk **a**kin **K**inase) due to its homology with murine Snk has been termed 'the odd one out of the family' (Sillibourne and Bornens, 2010). It is structurally divergent from PLK1-3 as it contains a single

carboxy terminal polo-box domain (PB3) and in place of the second polo-box it possesses a larger crypto polo-box domain (CPB), which includes two polo boxes (PB1 and PB2) and forms a bridge between the kinase domain and the carboxy-terminal Polo box (Carvalho-Santos et al., 2010; Slevin et al., 2012; Jana et al., 2012). (**Figure 3**)

The polo box domains which are characteristic of all members of the polo-like kinase family dictate the substrate specificity of the kinase and also regulates its function. PLK4, possessing a single polo-box compared to the two polo boxes of PLK1-3 indicates a different form of regulation for PLK4. The tandem polo-boxes of PLK1-3 form intramolecular heterodimers which then regulates the activity of the kinase by inducing a conformational change and allowing the catalytic domain gain access to its substrates (Elia et al., 2003). In contrast, the PLK4 polo box and its crypto polo box do not form heterodimers. Also, while the polo boxes of PLK1-3 are important for targeting the kinases to particular subcellular sites, deletion of the polo domain of PLK4 did not abolish subcellular localization to the centrosomes and only when the polo box and crypto polo box were removed did it fail to localize to the centrosomes. This reflects the presence of a second localization domain unique to PLK4 and shows that both the polo box and crypto polo box of PLK4 are independently able to localize PLK4 to its substrate (Leung et al., 2002). PLK4 also differs from the other polo-like kinases as it possesses three PEST sequences which are domains rich in proline(P), aspartate(D), glutamate(E), serine(S) and threonine(T) residues responsible for governing the stability of the protein. (Rechsteiner and Rogers, 1996). (**Figure 4**). The functions of the PEST motifs will be discussed shortly.

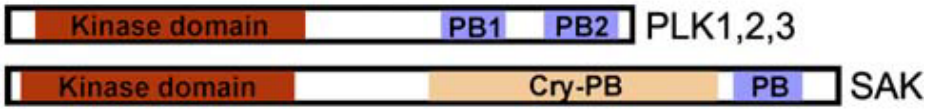


Figure 3: Domains of polo-like kinases. PB denotes polo box; Cry-PB denotes cryptic PB (Bettencourt-Dias et al., 2005)

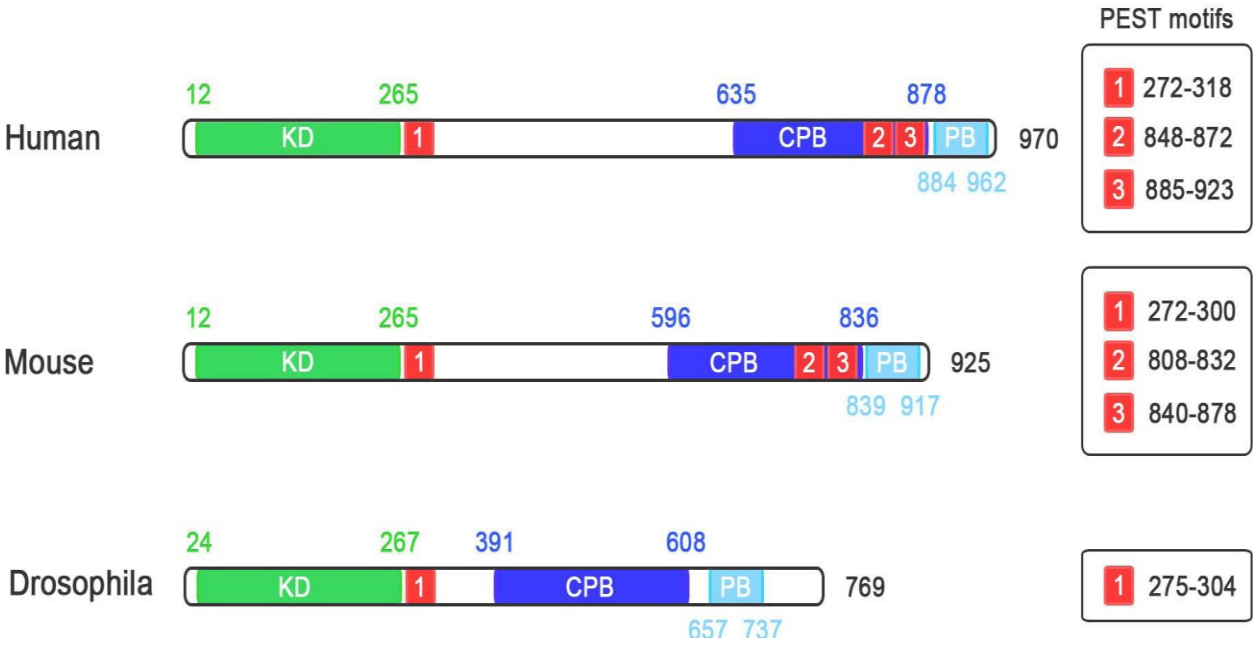


Figure 4: The structure of *Plk4* showing its PEST motifs in the numbered boxes (Sillibourne and Bornens, 2010)

POLO-LIKE KINASES –EXPRESSION, LOCALIZATION AND FUNCTIONS

The polo-like kinases show variations in abundance, structure, functions and localization at different stages of the cell cycle and are highly expressed in mammalian cells(**Figure 5)**. Jordan et al, 2012 showed that the expression of mRNA transcripts for PLK1-4 increase with progression of the first wave of spermatogenesis with lowest expression prior to meiotic entry (4 days post-parturm [dpp]) and reaching a maximum expression level by 16 dpp which corresponds to meiotic prophase (**Figure 6A**). They also show that PLK1-4 proteins were detected throughout the first wave of spermatogenesis, levels of PLK1-3 are relatively low prior to meiotic entry and increase when prophase to metaphase I (G2/MI) transition cells are present (19-22 dpp). PLK4 on the other hand show highest levels at 7-16 dpp and drop during the G2/M1 transition (19-22 dpp). Mixed germ cells from the adult testes show lower PLK4 protein levels, perhaps reflecting the lower representation of meiotic cells, and the faster migrating band may likely be a truncated or spliced variant form of PLK4 (**Figure 6B**).

Among the Polo-like kinases, PLK1 is the most characterized and is found in several locations throughout the mitotic cell cycle, whereas other polo-like kinases are restricted to specific stages and locations (Zitouni et al., 2014). PLK1 localizes to mitotic chromosome axes and centrosomes, centromeres, kinetochores, the spindle midzone in anaphase and the midbody in cytokinesis. PLK2 and PLK4 localize at or near the centrioles throughout the cell cycle and PLK3 localizes to the nucleolus (Abe et al., 2011; Archambault and Glover, 2009). Jordan et al, 2012 also show the localization pattern of

PLK1-4 by immunofluorescence microscopy assessment of spermatocyte nuclear spreads during meiosis I. At pachynema and diplonema, PLK1 signal is seen along the SC and by metaphase PLK1 is seen at the centromeres. PLK2 and PLK3 protein signals were observed throughout the chromatin in pachynema and diplonema while PLK4 signal localized to the sex body (**Figure 7**).

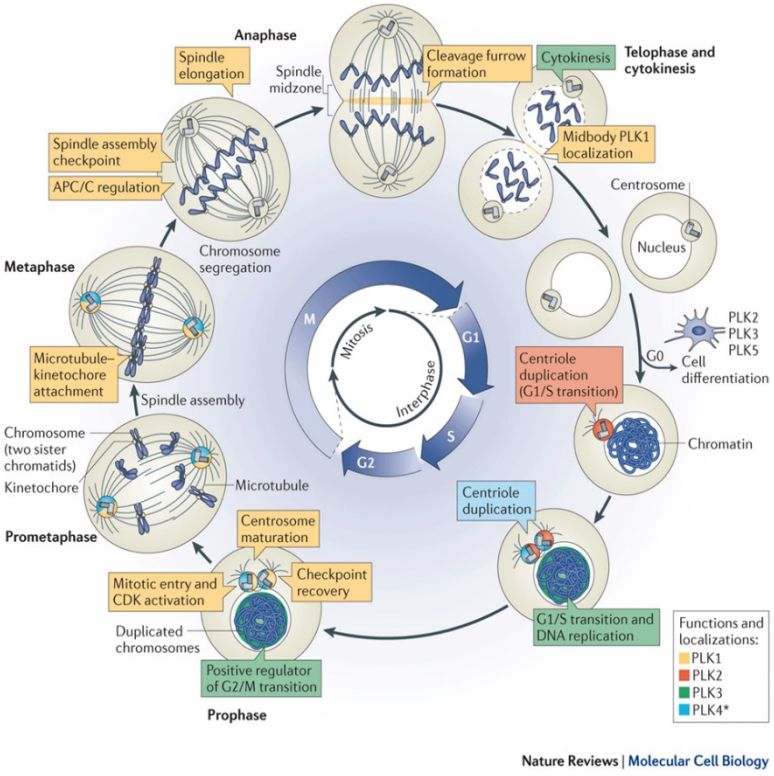


Figure 5: PLK functions and localizations during the mammalian cell cycle
(A) PLK1 functions begin in G2, where it localizes to the centrosomes. In prometaphase and metaphase, it is found at kinetochores and the spindle pole. It finally translocates to the central spindle in anaphase and telophase.
(B) PLK2 and PLK4 are localized to the centrosomes where they are involved in centriole duplication.
(C) PLK3 is implicated in DNA replication, G1/S transition, G2/M transition and cytokinesis. (Zitouni et al., 2014)

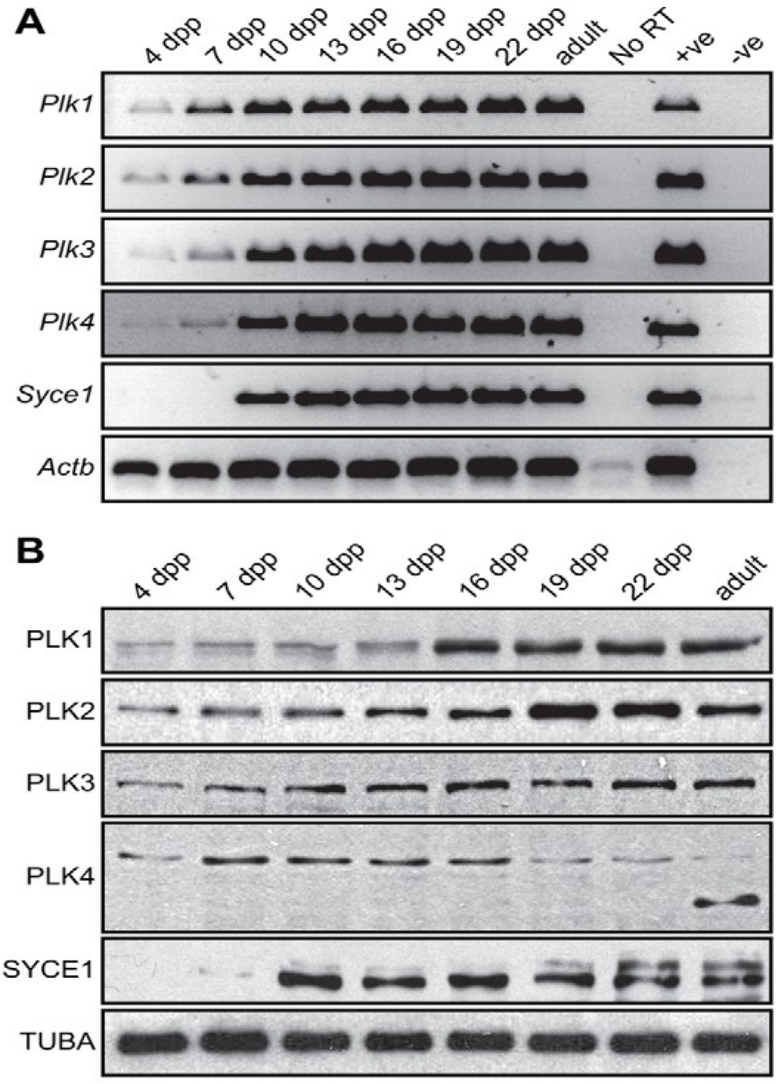


Figure 6: Expression of polo-like kinases during the first wave of spermatogenesis
(A) mRNA expression for PLK1-4; syce1 was used as control for progression of the first wave of spermatogenesis; Actb was used as mRNA loading control
(B) Protein expression for PLK1-4; syce1 was used as control for the progression of the first wave of spermatogenesis; TUBA was used as protein loading control. (Jordan et al., 2012)

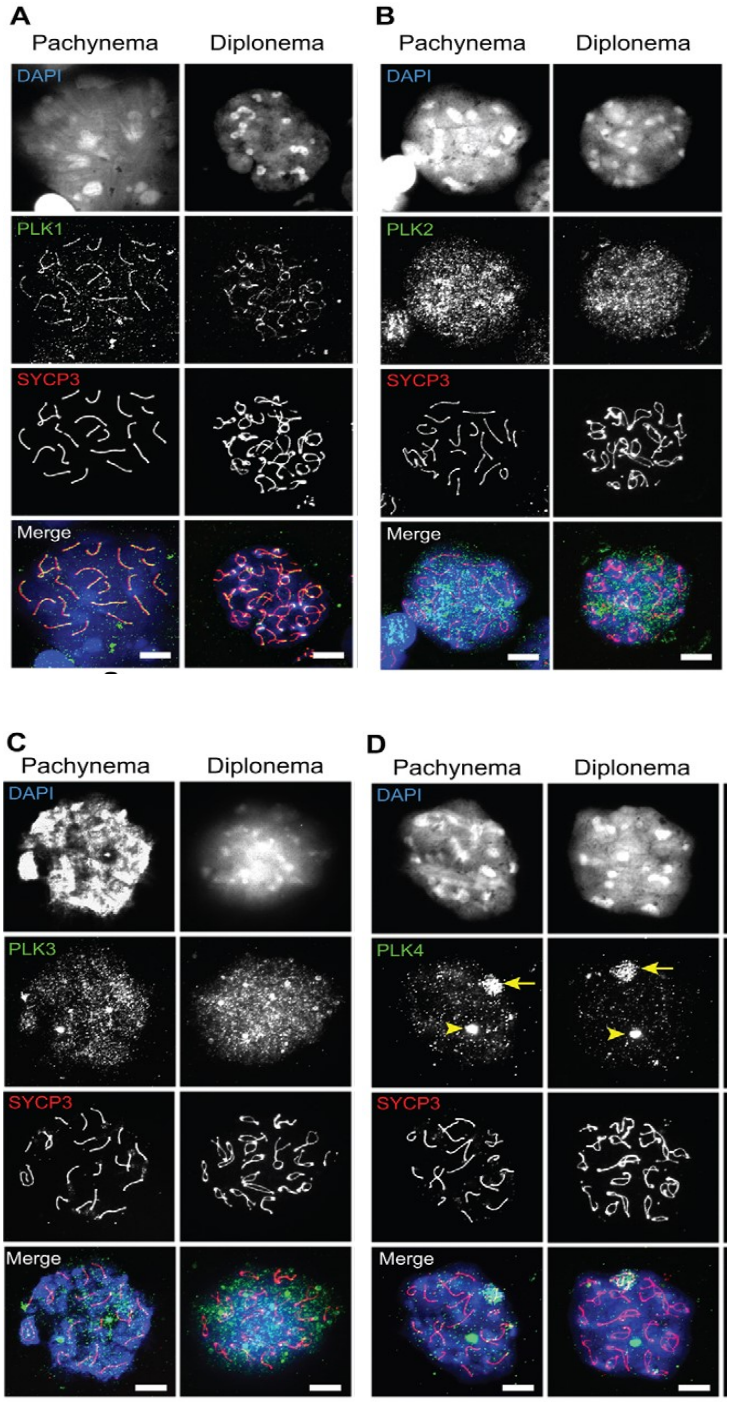


Figure 7: Nuclear spreads showing localization patterns for PLK1-4 during pachytene and diplotene stages of meiotic prophase respectively. (Jordan et al., 2012)

PLK4 AND THE CENTROSOME

Centrosomes are the major microtubule organizing center (MTOC) of animal cells and their accurate number is critical to genome integrity. Centrosomes comprise of a pair of centrioles arranged orthogonally and centriole duplication controls centrosome duplication which occurs once per cell cycle. As mentioned earlier PLK1, PLK2 and PLK4 localize to the centrosomes and although PLK1 and PLK2 are involved in regulating centriole duplication, centrioles that are MTOC competent (referring to the ability of centrioles to recruit pericentriolar materials and nucleate microtubules) when disengaged from daughter centrioles can support duplication irrespective of whether PLK1 is present or not (Wang et al., 2011). *Plk2*-knockout mice are also viable, suggesting that its function can be substituted by other polo-like kinases (Ma et al., 2003). PLK4 on the other hand associates with the centrioles throughout the cell cycle. Its overexpression as shown in various studies leads to increased centriole numbers and its depletion causes progressive reduction in centriole numbers (Habedanck et al., 2005). Hence, PLK4 is key to the process of centriole formation and has been termed the master regulator of centriole biogenesis. PLK4 is indispensable to cells as indicated by its presence in actively dividing tissues and *PLK4* knockout embryos arrest at stage E7.5 (Hudson et al., 2001).Research on PLK4 has focused on its canonical centrosome function and its substrates are still being investigated.

PLK4: CONTROL AND REGULATION

To ensure that centriole duplication does not go awry and to maintain genome integrity, PLK4 abundance in the cell must be tightly controlled and regulated. PLK4 is a low abundance kinase whose stability is directly linked to the activity of the enzyme, its expression is controlled transcriptionally with its mRNA expression increasing from interphase and reaching a peak during mitosis (Fode et al., 1996) and meiosis (Jordan et al., 2012). In mouse cells *Plk4* mRNA levels are downregulated by the p53-DREAM signaling pathway. Nuclear factor-κB (NF-κB) has also been shown to activate PLK4 expression (Ledoux et al., 2013). The PEST sequences also govern PLK4 protein stability (**Figure 8**). One PEST sequence is located within the amino terminus and two others within the carboxy terminus- all of which play a part in the turnover of the kinase (Yamashita et al., 2001). The first PEST sequence contains a degron motif, which is conserved all through evolution (**Figure 8**). Phosphorylation of the serine and threonine residues in the degron motif generates a binding site for the ubiquitin ligase SCF (SKP1-CUL1-F-BOX) complex (Cunha-Ferreira et al., 2009). This SCF complex ubiquitinates PLK4 and it is targeted to the proteasome for destruction (Rogers et al., 2009).

Finally, PLK4 has a short half-life of about 2-3 hours in the cell (Fode et al., 1996). Several studies have shown that the kinase activity of PLK4 autoregulates its own stability. Inactivation of the kinase activity of PLK4 has been shown to stabilize the protein accumulating up to > 10 fold higher levels than the active kinase, suggesting that active PLK4 stimulates its own breakdown (Peel et al., 2012). The SCF E3 ubiquitin ligase

has been found to localize to the centrosomes associating with substrates phosphorylated at specific sites in phosphodegron motifs (Klebba et al., 2013). The speculated mechanism of PLK4 degradation by autophosphorylation is depicted in **Figure 9**. Two PLK4 monomers homodimerize through their CPB domains and the intertwining of PB3 domains, the kinase domain also becomes active through auto-phosphorylation. This is followed by transphosphorylation of the degron which activates a negative feedback pathway promoting its degradation through the ubiquitin–proteasome pathway (Jana et al., 2012; Sillibourne et al., 2010). The degron phosphorylation is dependent on autophosphorylation of sites around it (Holland et al., 2010), however, this multisite trans-autophosphorylation around the degron ensures that the PLK4 protein phosphorylation attains threshold before its autodestruction (Cunha-Ferreira et al., 2013). This phosphorylation-dependent degradation of PLK4 is counteracted by protein phosphatase 2A (PP2A) which stabilizes PLK4 during mitosis (Brownlee et al., 2011). Worthy of note however is that PLK4, though reported to be a low abundance kinase is highly expressed during spermatogenesis and this suggests a possible different mechanism of regulation for PLK4 during spermatogenesis.

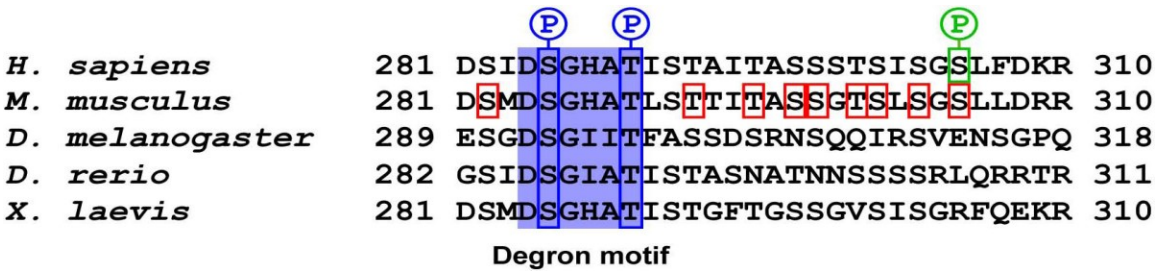


Figure 8: The degron motif of PLK4 (highlighted in blue) conserved in various species; potential sites for autophosphorylation (highlighted in red); S305, conserved and autophosphorylated in *H. sapiens* (highlighted in green)

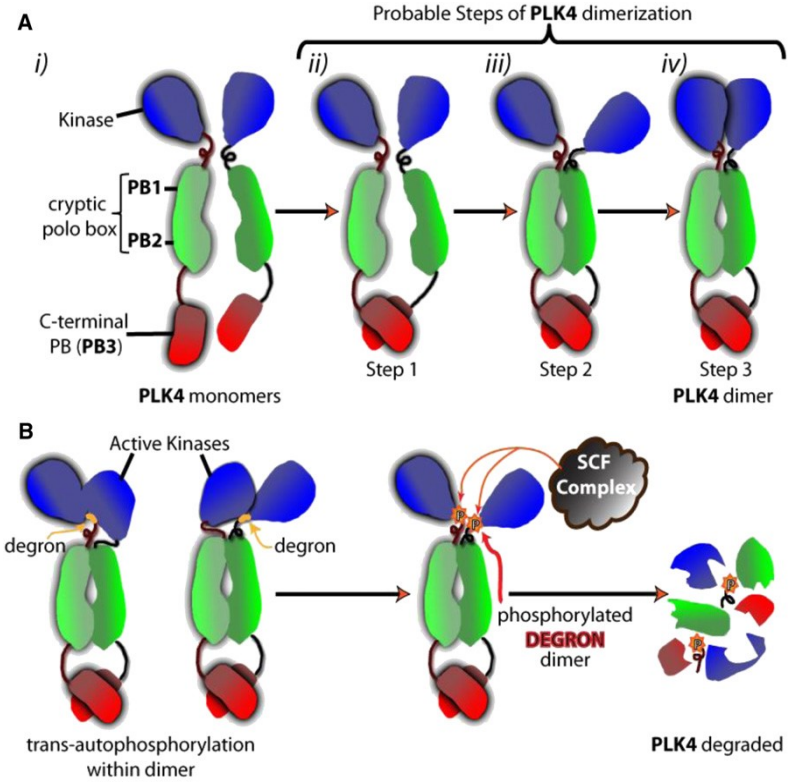


Figure 9: Speculative model for PLK4 dimerization, tans-autophosphorylation of the degron motif, recognition of the phosphorylated degron motif by the SCF complex and eventual degradation of the PLK4 molecule (Jana et al., 2012)

PLK4 AND SPERMATOGENESIS

PLK4 research, though largely centered on its canonical role in centriole biogenesis, it has recently been implicated to have novel roles in spermatogenesis. In a screen for ENU-induced mutations that cause male hypogonadism, Harris et al, (2011) identified a novel heterozygous missense mutation in the kinase domain of PLK4, altering an isoleucine to asparagine at residue 242 (I242N). This study demonstrated that PLK4 is required for proficient spermatogenesis. Histologically, the affected animals show discrete regions of abnormal seminiferous tubules lacking in germ cells which they describe as patchy germ cell loss observed from postnatal day 10 and subsequent stages, their testis size was also reduced by 17%. In another study carried out by Miyamoto et al., (2015) where they carry out mutational analysis of 81 patients with azoospermia and sertoli cell-only syndrome (SCOS), they identified one man with a 13-bp deletion in the Serine/Threonine kinase domain of PLK4 also implicating a possible role for the protein in spermatogenesis. Furthermore, our lab discovered that PLK4 localized to at the sex body during pachynema and diplonema of meiotic prophase I and its localization pattern is similar to other proteins such as ATR, BRCA1 and γH2AX that are involved in DNA damage response (DDR) and meiotic sex chromosome activation (MSCI). However, a specific role for PLK4 in mammalian spermatogenesis is yet to be discovered. My project aims to assess the effects of inhibiting PLK4 on meiotic progression during the first wave of spermatogenesis in mammalian germ cells, using mice as a research model.

PLK4 AND MSCI

Having established that PLK4 localizes to the sex body during prophase I of meiosis, its localization pattern resembles other proteins such as ATR, BRCA1 and γH2AX that are involved in DNA damage response (DDR) and meiotic sex chromosome activation (MSCI). Defective synapsis is recognized by the pachytene checkpoint via DNA damage repair factors such as ATR and BRCA1, the homologs that fail to synapse are transcriptionally inactivated in a chromosome wide phosphorylation of H2AX (γH2AX) in a process known as meiotic silencing of unsynapsed chromosomes (MSUC). Persistent autosomal asynapsis is thought to trigger apoptosis by starving the cells of essential gene products (Royo et al., 2010, 2013). During male meiosis, neither sex chromosome has a homolog. However, the largely heterologous X and Y (sex) chromosomes do pair and synapse along a spatially restricted region called the pseudo-autosomal region (PAR) which is where recombination occurs. The unsynapsed region of the sex chromosomes undergoes remodeling of the chromatin into heterochromatin, leading to transcriptional inactivation and compartmentalization of the sex chromosomes into a specialized and visible domain called the sex body within the nucleus of pachytene-staged spermatocytes. This process of transcriptionally silencing of the heterologous X and Y (sex) chromosomes during prophase I of male meiosis is called meiotic sex chromosome inactivation (MSCI) (**Figure 10**) and is thought to be a more complex manifestation of MSUC. Errors in chromosome synapsis have long been associated with chromosomal anomalies and infertility (Harton and Tempest, 2012). Sex chromosome abnormalities e.g. Klinefelter (XXY) syndrome constitutes the largest class of

chromosome abnormalities and the commonest genetic cause of infertility in humans, approximately one-half of such cases results from non-disjunction of X and Y homologs at the first meiotic division (Thomas and Hassold, 2003; Heard and Turner, 2011). The escape from MSCI is also thought to be directly responsible for pachytene arrest on XYY mice and men which leads to germ cell loss and eventual infertility (Turner et al., 2006; Mahadevaiah et al., 2000). MSCI failure therefore leads to apoptosis and the process is indispensable for male fertility (Royo et al., 2010, 2013). The mechanistic details and various players involved in MSCI are still yet to be fully elucidated and PLK4 may very likely play a role in the process.

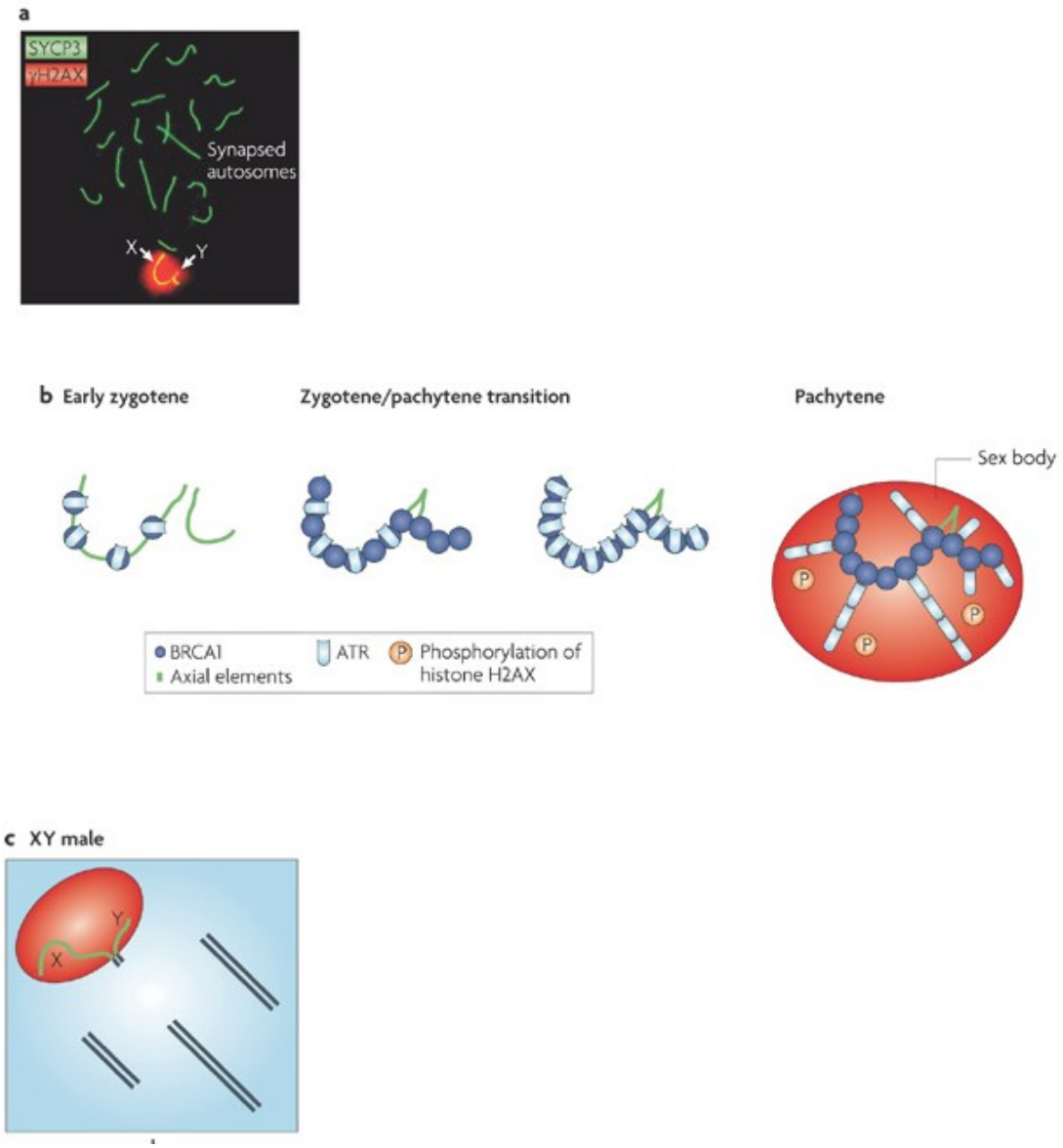


Figure 10: An overview of MSCI. **A**- Pachytene spermatocytes stained for SYCP3 and γH2AX which marks the transcriptionally silenced domain (the sex body). **B**- At the beginning of zygotene BRCA1 and ATR are present at the sites of double stranded breaks (DSBs). At zygotene/pachytene transition there is further recruitment of BRCA1 and ATR which phosphorylates H2AX triggering changes leading to MSCI. **C**- Schematic of XY pachytene spermatocytes with three fully synapsed bivalents and the asynapsed region of the XY located in the transcriptionally silenced sex body. (Burgoyne et al., 2009)

CURRENT PROJECT AND HYPOTHESIS

Having established the possibility of an active role of PLK4 in spermatogenesis, we hypothesize that inhibiting the kinase activity of PLK4 may lead to aberrant DNA repair process, chromosome missegregation, disruption in the meiotic process and eventual loss of germ cells. Due to the similarity of the kinase domains in PLK1-4, their sensitivities to small molecule inhibitors are similar. Kinase inhibitors such as MLN8054 have been previously reported to target numerous kinases including PLK4 but with greater specificity for Aurora A and B (Karaman et al., 2008). Another inhibitor VX-680, a dual Aurora A/B inhibitor (Girdler et al., 2008) has also been shown to inhibit PLK4 with IC_{50} of <0.3µM in the presence of 100µM competing ATP (Sloane et al., 2010).

Using VX-680 as a template to create a selective PLK4 inhibitor, Wong et al., (2015) introduced a methoxy substituent at the VX-680 C5 position and generated a compound with ~15-fold preference for PLK4 over Aurora A. Approximately 34% of the analogs they synthesized had half-maximal inhibitory concentration (IC_{50}) values ≤ 100nM for PLK4 in vitro. Only one of those analogs depleted centrosomes in NIH/3T3 mouse embryonic fibroblasts at concentrations <10µM, this analog was optimized to produce two highly selective PLK4 inhibitors that were named - Centrinone (centrosome depletion at 100nM) and Centrinone B (centrosome depletion at 500nM). Both Centrinones were reported to exhibit >1000 selectivity for PLK4 over Aurora A/B in vitro and did not affect cellular Aurora A or B.

To show the selectivity of Centrinone for PLK4 over Aurora A and B, they treated HeLa cells with DMSO, VX-680 (1µM) and Centrinone (125nM) for 7 hours and then treated with reporters of Aurora A and B activity. The results show same phenotype for Centrinone and DMSO with the expected inhibitory effect from VX-680. Finally, shown below are two tables reflecting the binding affinity of Centrinone for PLK4 and other kinases.

Table 1 - K_i values of centrinone, centrinone B and VX-680 for PLK4 and Aurora A/B

KINASES	K_i (nM)		
	Centrinone	Centrinone-B	VX-680
PLK4	0.16	0.59	7.66
AURORA A	171.00	1239.00	0.65
AURORA B	436.76	5597.14	3.36

Table 2 - K_i values of centrinone, centrinone B and VX-680 for PLK4 and other kinases

KINASES	K_I(nM)	
	Centrinone	Centrinone-B
PLK4	0.16	0.59
TNK1	1.38	6.04
LRRK2	2.08	7.66
ROS/ROS1	2.77	90.07
FLT4/VEGFR3	6.29	58.04
RET	13.92	195.25
JAK2	>150	>500
SRPK1	>150	>500

Thus far, PLK4 has only been studied within the context of its centrosome function with no information about its role in spermatogenesis. As previously stated, mutation in the kinase domain of PLK4 has been associated with germ cell loss and PLK4 localizes to the sex body during meiotic prophase I (Harris et al., 2011; Jordan et al., 2012), and so we hypothesize that PLK4 is required for chromosomal segregation and the normal progression of MSCI and inhibiting PLK4 will lead to abnormalities in meiotic progression. To test this hypothesis, we extract pachytene-staged spermatocytes from mice at 17-19 dpp which are undergoing first wave of spermatogenesis, the cells are cultured in spermatocyte culture media before the addition of centrinone, the small molecule reversible inhibitor of PLK4. We assess meiotic progression, chromosome synapsis, sex body formation, DNA damage and its response proteins while inhibiting PLK4. Further analyses were done on the ENU mice carrying a heterozygous mutation for PLK4 (*Plk4*$^{+/I242N}$). We report that PLK4 indeed localizes to the sex body during meiotic prophase I, centrinone specifically inhibits PLK4, delayed meiotic progression and we show evidence of a DNA damage response observed while inhibiting PLK4. We also report that RNA Polymerase II (RNA Pol II) which is normally excluded from the sex body indicating normal MSCI is not robustly excluded in the mutant. These findings provide and promote the evidence that PLK4 is required to ensure accurate chromosome segregation and normal progression of MSCI in the early spermatogenesis.

MATERIALS AND METHODS

ENU MUTAGENESIS

Plk4$^{+/I242N}$ mice were generated by administering the chemical mutagen N-nitroso-N-ethylurea (ENU) to mice via intraperitoneal injection as described by Siekpa and Takahashi, 2005. Mutagenesis was performed on male mice of the C57BL/6J (B6) strain, ENU-treated males (G0 generation) were allowed 12 weeks to recover fertility and were then crosses to untreated B6 females yielding G1 generation males and females. For the cross designed to detect dominant mutants, G1 males and females were bred to wild type DBA mice and the resulting F1 male progeny screened. For the cross designed to detect recessive mutants, G1 males were bred to wild type B6 females and the resulting G2 females were back-crossed to their G1 fathers yielding G3 male pprogeny for screening. Mice were monitored and screened for urogenital morphology, testis weight measurement and testicular histology. Mice were killed at postnatal (P) 1, P10, P15, P21, P31, and 6 and 8 weeks of age, testis and body weights were recorded for all postnatal animals and photographs of the testes of wild type and mutants were taken at 6 weeks. All experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Animals. (Harris et al., 2011; Weiss et al., 2012).

POLYMERASE CHAIN REACTION (PCR), PCR CLEANUP AND SEQUENCING

Genotyping of wild type and mutant mice was done by PCR. DNA was extracted from mice by digesting tail tips first with 50 mM NaOH, followed by centrifugation and boiling to completely dissociate the tissue. This is then neutralized with 1M tris HCl (pH 8) and serves as DNA template for the PCR reaction. Each PCR reaction contained a total volume of 50µl, consisting of molecular grade water, 10x buffer, dNTPs, DNA polymerase, primers and DNA from sample (**Table 3**). The following PCR conditions were used:

1. Denaturation: 90^0C for 1 minute
2. Denaturation: 90^0C for 20 seconds
3. Annealing: 58^0C for 30 seconds
4. Amplification: 72^0C for 1 minute
5. Steps 2-4 were repeated for 30 cycles
6. Final extension: 72^0C for 10 minutes

After PCR completion, 10 µl of each sample was run on a 2% agarose gel to check if the PCR worked before proceeding to a PCR cleanup. Cleanup was done using the isolate II PCR and gel kit (Bioline) containing binding buffer, wash buffer and elution buffer. Finally, 10 µl of each cleaned up PCR reaction is added to 5 µl of the primer sequence -CGAAGTGCACATGGACTTGAAT- and sent for sequencing. The sequence ACCTTAT indicates the wild type sequence while ACCTTAT/A indicates the heterozygous mutant sequence (**Figure 11**).

Table 3: PCR setup for 4 samples

REACTION	VOLUME/REACTION (µl)	MASTER MIX VOL (µl)
H_20	38.2	152.8
10x buffer	5	20
DNTPs	0.4	1.6
DNA polymerase	0.4	1.6
Primer mix 471 + 474	1	8
Total		184
Master mix per sample		46
DNA per sample		4

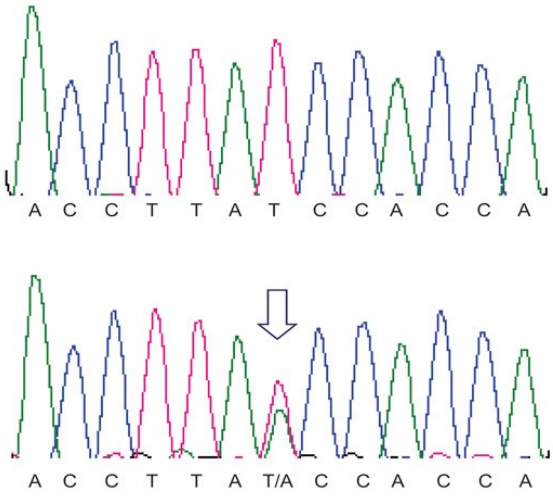


Figure 11: The sequence of the wild type (*Plk4*$^{+/+}$) shown above and the ENU heterozygous mutant (*Plk4*$^{+/I242N}$) shown below (Harris et al., 2011)

GERM CELL EXTRACTION AND MICROSPREADS

Germ cells were isolated from mice at 17-19 days post-partum (dpp) which are enriched in mid-prophase spermatocytes (2.5 x 10^6 cells/ml). Mice were euthanized via cervical dislocation, and both testes were removed and placed in 1x phosphate buffered saline (PBS). The tunica albicans was then removed and detunicated testes placed in Krebs-Ringer bicarbonate solution (KRB) supplemented with protease inhibitor (PI) cocktail solution (Roche) to prevent degradation of proteins and liberate germ cells. The testes is shredded using fine forceps and solution allowed to sit in 5ml KRB for a few minutes, then filtered using 0.8µm Nitex mesh and allowed to centrifuge at 2500 rpm for 7 minutes at 4^0C. The supernatant is discarded, the cells resuspended with spermatocyte culture media and left to equilibrate for 1-2 hours in the incubator (32^0C) before the addition of inhibitors. To inhibit PLK4, 5µM centrinone was used; 5µM ZM447439 was used to inhibit Aurora kinases, 5µM okadaic acid (OA) was used to induce prophase to metaphase (G2/MI) transition. DMSO and ethanol were used as control.

Cells are collected at the various time points set for each experiment, centrifuged at 9000rpm for 5 minutes, supernatant discarded, cells resuspended with 0.1M sucrose solution, and placed on slides incubated with 1% PFA solution. Slides were allowed to sit for 2-2.5 hours in a dark moist chamber, rinsed in 1x PBS + 0.2% photoflo solution, left to air dry and then washed 3x in wash buffer (1x PBS with Antibody

Dilution Buffer [ADB]) before the addition of primary antibodies. Solution compositions are shown below (Table 3).

STAINING AND MICROSCOPY

Primary antibodies were placed on slides and left to incubate at 4^0C overnight. Primary antibodies against the following proteins were used: Goat anti-SYCP3 (1:50 dilution), Rabbit anti-PLK4 (1:200 dilution), Rabbit anti-SYCP1 (1:1000 dilution), Mouse anti-RNA Pol II (1:400 dilution), Mouse anti-γH2AX (1:400 dilution), Goat anti-ATR (1:50 dilution), Rabbit anti-RAD 51 (1:100 dilution). After incubating with primary antibodies, slides are rewashed in 1x PBS + photoflo and wash buffer steps repeated in preparation for secondary antibodies. These primary antibodies were detected with secondary antibodies raised against mouse, rabbit and goat and conjugated to Alexa 488, 555 and 647 (life technologies) at 1:500 dilution and then left to incubate for 1-2 hours in the dark moist chamber at 37^0C.

After incubation with secondary antibodies, slides are washed 2x in 1x PBS + 0.2% photoflo and 2x in water + 0.2% photoflo (pH 8.0). Spreads are then mounted with Vectashield + DAPI medium (Vector Laboratories) and images captured using an Axio cam ERc5s Zeiss fluorescence microscope (Hamamatsu).

HISTOLOGY

Tissues from the testis was fixed in bouins fixative then washed in 70%, 90% and 100% ethanol to dehydrate the tissue. Tissues were then embedded in paraffin and serial sections 5 microns thick (5µm) were placed onto slides and stained with hematoxylin and eosin (H&E).

IMAGE AND STATISTICAL ANALYSIS

Images were processed and analyzed using the Zeiss Zen 2012 blue edition image software. Further analysis was completed using Adobe Photoshop CS6 and the Image J software provided by the National Institute of Health. Data was then exported to Microsoft Excel and standard statistical analysis was run via GraphPad Prism.

TABLE 4 – SOLUTIONS

SOLUTION	INGREDIENTS
Krebs-Ringer Bicarbonate Media	• 39.0ml of 9% NaCl • 15.6ml of 1.15% KCl • 16.3ml of 6.5% $NaHCO_3$ • 3.9 ml of 2.1% KH_2PO_4 • 3.9ml of 3.8% $MgSO_4 .7H_2O$ • 5.9ml of 1.2% $CaCl_2$ • 5.0ml of 100x Penicillin/Streptomycin/Glutamine • 5.0ml of 100x non-essential aa • 50x MEM essential aa
Spermatocyte Culture Media	• 50ml MEMα • 145µl Na lactate • 2.5ml FBS • 0.295g Hepes
Sucrose (0.1M), 5ml	• 1ml of 0.5M Sucrose • 4ml o MQ H_20 • 100µl of 50mM NaOH (to bring the $_pH$ to 8) • 20µl of 50x protease inhibitor cocktail (Roche)
PFA (1%), 5ml	• 312.5µl of 16% PFA • 4687.5 MQ H_20 • 60µl of 50mM NaOH (to bring the $_pH$ to 8) • 20µl of 50x protease inhibitor cocktail (Roche) • 50µl of 10% Triton X-100
Antibody dilution buffer (ADB)	• 50ml of PBS • 3% BSA • 10% goat or horse serum • 0.05% Triton X-100
Washing/Blocking buffer (WB) (10% ADB)	• 45ml of PBS • 5ml of 100% ADB

RESULTS

PLK4 SIGNAL LOCALIZES TO THE SEX BODY DURING PACHYNEMA AND DIPLONEMA

The localization of PLK4 to during meiosis prophase I as reported previously was confirmed by chromosome spread preparations (**Figure 12**). PLK4 localizes to the largely unsynapsed X and Y chromosome pair during prophase I of male meiosis resembling the localization of other protein known to be involved in MSCI such as ATR, DMC1 and γH2AX (the phosphorylated form of H2AX and also a marker for meiotic double strand breaks). PLK4 has also been observed to localize at unsynapsed autosomal axes in wild type and abnormal cells bearing Robertsonian translocations (data not shown). Using antibodies against the lateral element protein of the synaptonemal complex SYCP3 as a marker to stage the progression of meiosis, wild type mice at 17-20 days post-partum (dpp) are usually enriched in pachytene and diplotene stage cells. At pachynema, all autosomes are fully synapsed as indicated by linear SYCP3 signal while the X and Y chromosomes are synapsed only at the pseudoautosomal region (PAR) and have compartmentalized to form the sex body. At diplonemaa, desynapsis has begun and this is marked by disassembly of the SC central elements, the sex chromosomes however remain compartmentalized in the sex body domain. PLK4 signal is shown at the sex body during pachynema and diplotenema confirmed by colocalization with γH2AX (**Figure 12**).

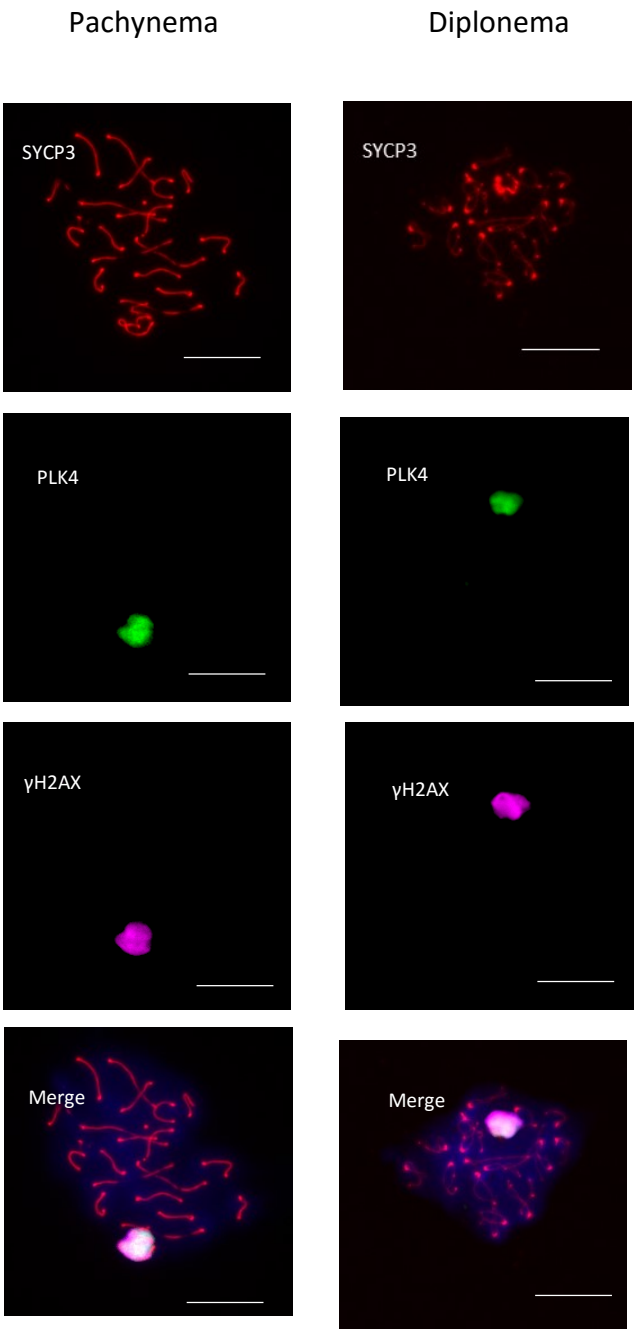


Figure 12: Localization of PLK4 in the male germ cell. Chromosome spreads from primary spermatocytes of B6 wild type mice aged 18 dpp were immunolabeled using antibodies against SC lateral element protein SYCP3 (red), PLK4 (green), γH2AX (far-red). Scale bars: 10µm.

OPTIMIZATION OF CENTRINONE TO MAXIMALLY INHIBIT PLK4

Wong et al., (2015) developed the small molecule inhibitor, centrinone to selectively inhibit PLK4 and reversibly deplete centrioles from cells. The authors used 125nM and 300nM concentrations of centrinone to inhibit PLK4 in various cell lines including HeLa and NIH/3T3. To determine the optimal concentration of centrinone to use for the male mouse primary spermatocytes, a time course experiment was carried out using different concentrations of centrinone for various times. Having mentioned that PLK4 is expressed at high levels during spermatogenesis, higher concentrations than were necessary to deplete centrioles in cell lines were tested. Concentrations tested were 500nM, 1µM, 1.25µM, 2.5µM, 5µM, 10µM and 20µM for 6 hours, 12 hours and overnight (20 hours) respectively. Cells were assessed based on the quality of the spread and number of cells with γH2AX signal present throughout the chromatin (**Figure 13**). Cells treated with 10µM and 20µM showed very low quality spreads and so treatment with 5µM centrinone for 20 hours was chosen for the experiments (**Figure 14**). To further prove the specificity of centrinone for PLK4 and not Aurora kinases, experiments were done comparing the effect of centrinone and ZM447439 (Aurora kinase inhibitor) during meiotic prophase I. The results demonstrated that cells treated with ZM447439 did not display the widespread localization of γH2AX typically seen with centrinone treatment (**Figure 15A**). Furthermore, with the addition of okadaic acid, a phosphatase inhibitor known to induce the prophase to metaphase transition, cells treated with ZM447439 arrest at diplotene while centrinone treated cells progress further towards metaphase (**Figure 15B**).

Wild type (normal) + centrinone (abnormal)

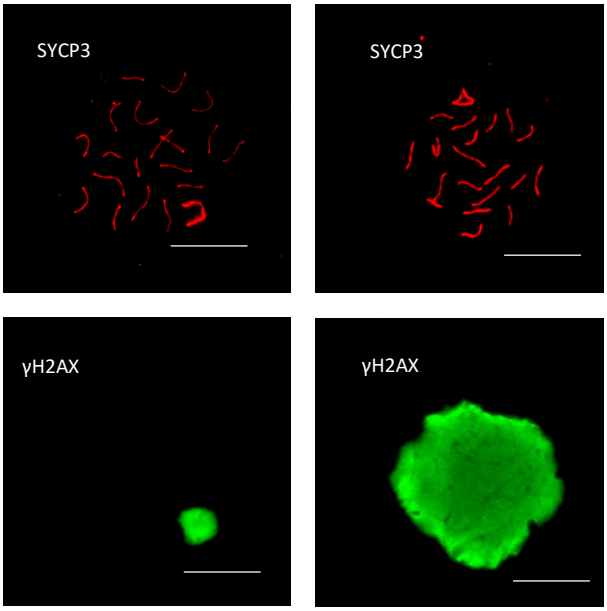


Figure 13: γH2AX signal located on the sex body of wild type cells and present throughout the chromatin of pachytene-staged centrinone treated cells

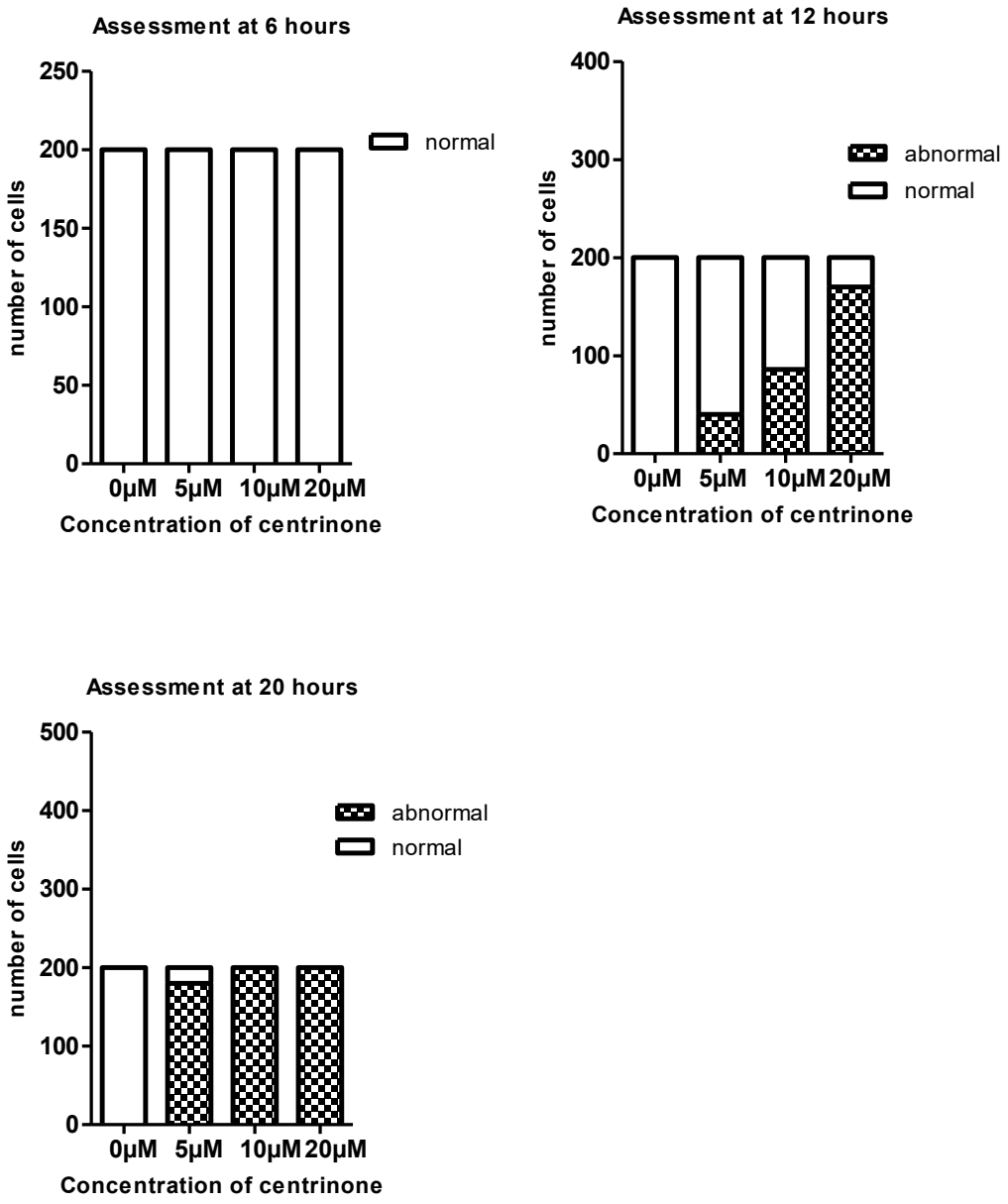


Figure 14: Time course experiments with different concentrations of centrinone at various time points to choose an optimal effective concentration of centrinone for the male mouse primary spermatocytes. Assessment of cells after 6 hours of inhibition with centrinone show the normal phenotype similar to the wild type cells, 12 hours inhibition show a mix of normal and abnormal cells and at 20 hours all cells at 10μM and 20μM are abnormal. Cells were treated with 5μM centrinone for 20 hours.

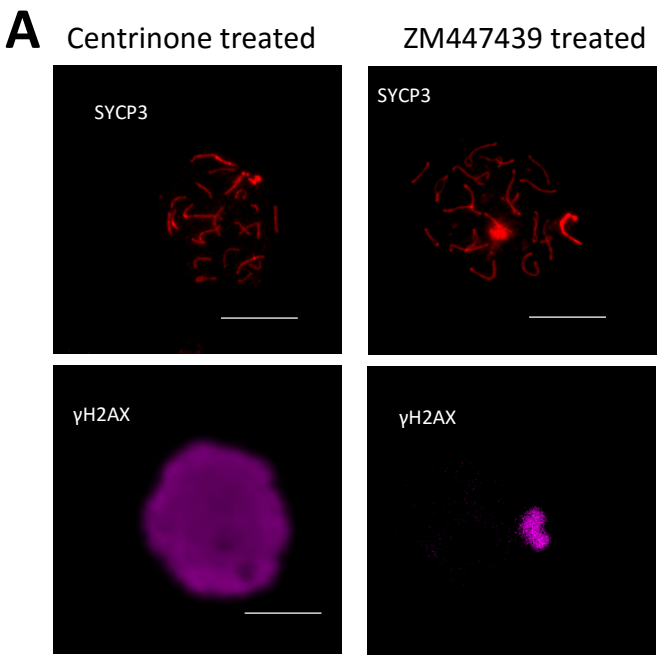


Figure 15A: Delineating the effects of centrinone from other kinase inhibitors, treatment with centrinone shows widespread γH2AX signal but γH2AX signal is limited to the sex body in ZM447439 treated cells.

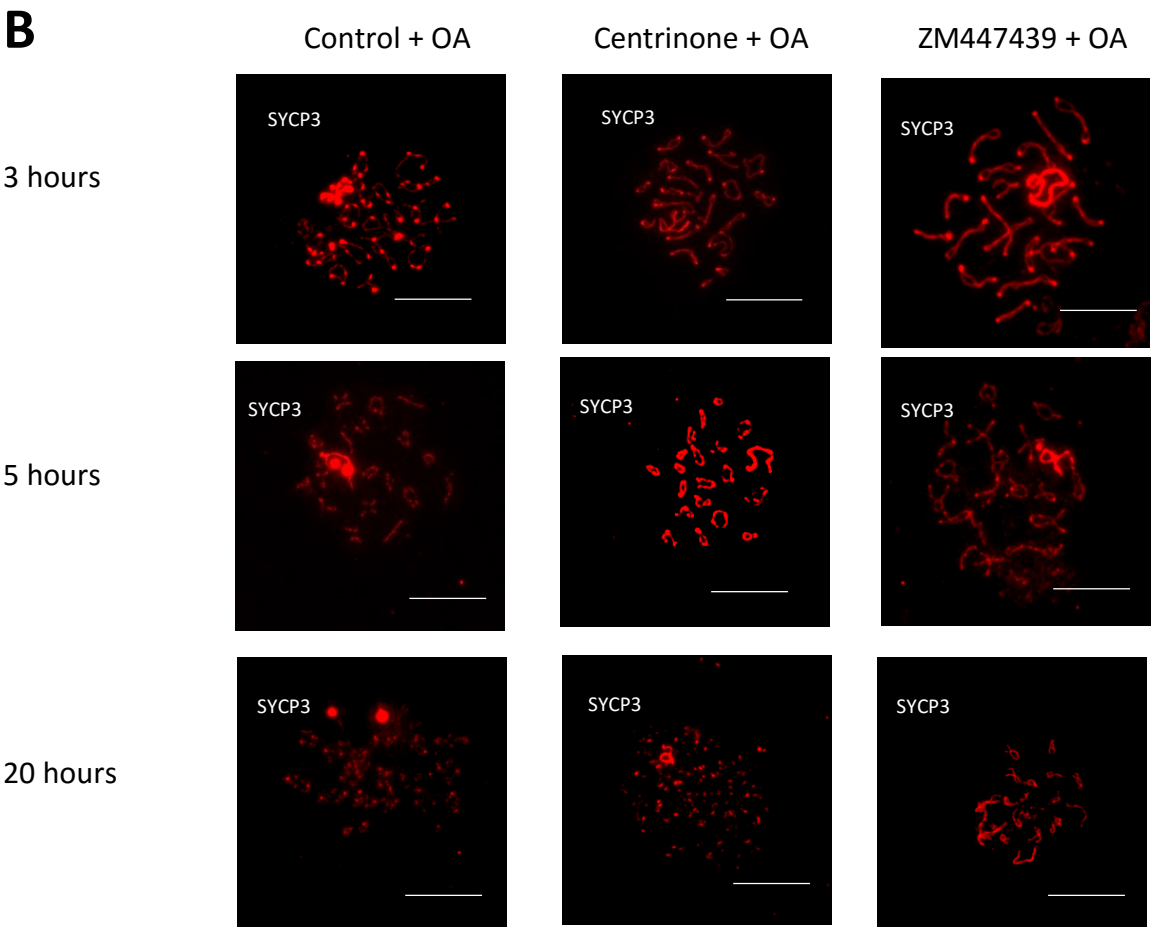


Figure 15B: Delineating the effects of centrinone from other kinase inhibitors, addition of okadaic acid initiates exit from meiotic prophase as indicated by the disassembly of lateral element protein SYCP3. At 3 hours all cells are at diplotene stage, ZM447439 treated cells arrest at this stage while centrinone and wild type progress further. The phenotype described is observed in all cells (N=200), Scale bars: 10µm

PLK4 INHIBITION WITH CENTRINONE SHOWS DNA DAMAGE PHENOTYPE

The phosphorylation of H2AX to form γH2AX is one of the earliest cellular responses to the formation of double strand breaks initiated by the meiosis specific topoisomerase II-like enzyme, SPO11 (sporulation 11) during leptonema of meiotic prophase I. As meiosis progresses, the loss of γH2AX occurs in conjunction with synapsis and at pachytene, γH2AX signal is completely absent at the autosomes and present only at the sex body (Mahadevaiah et al., 2001). We assessed pachytene stage cells after the addition of 5μM centrinone overnight using immunofluorescence microscopy and we observed several meiotic aberrancies. Most prominent was the spread of γH2AX signal present throughout the chromatin in ~ 80% of the cells. To confirm that this was due to the inhibition of PLK4 and not toxicity from centrinone, washout experiments were carried out by replacing centrinone treated media with fresh spermatocyte media 3x for 3 hours respectively and all these cells show similar phenotype to the wild type cells (**Figure 16A**). Furthermore, we asked if the γH2AX signal was due to the presence of new double strand breaks and used antibodies to Radiation sensitive 51 (RAD51), a strand invasion protein to assess that. RAD51 foci was clearly seen on both autosomes and sex chromosomes in about 20% of the cells at pachytene stage indicating a possible new round of DNA damage in response to PLK4 inhibition(**Figure 16B**).

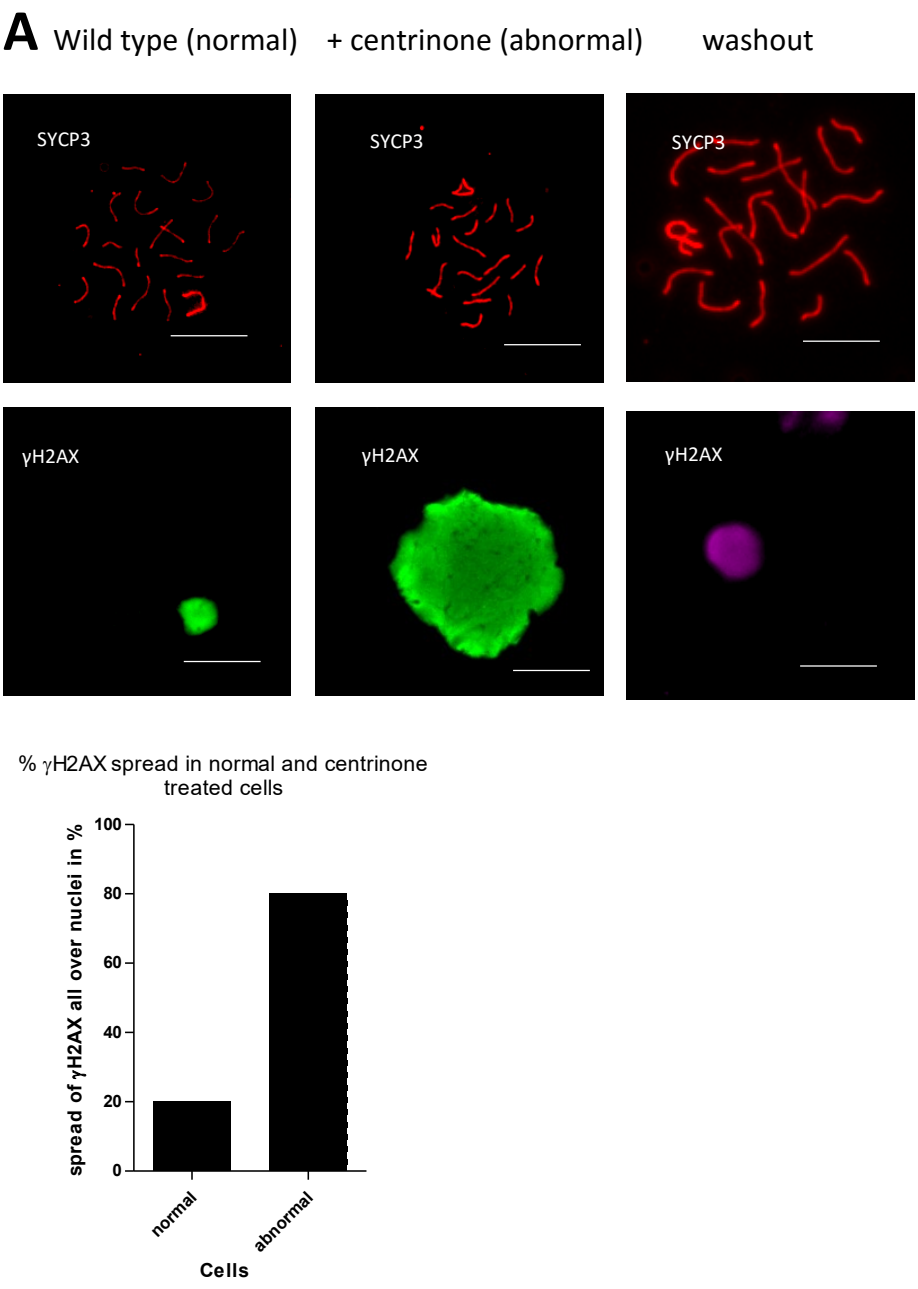
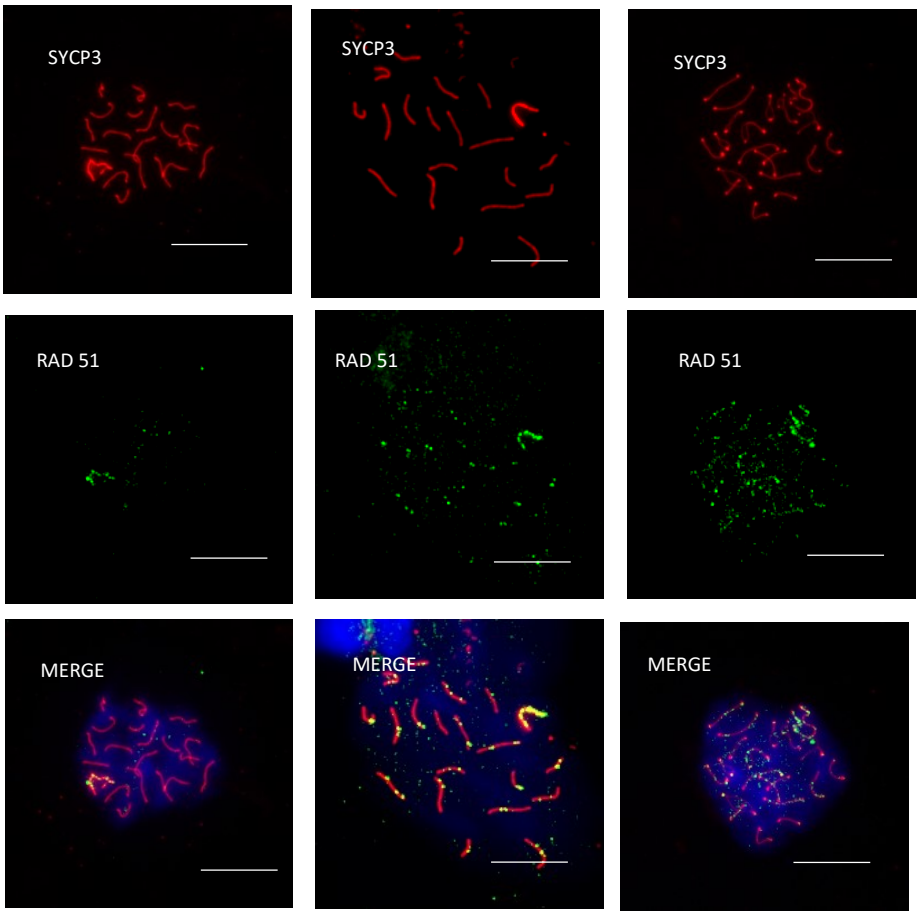


Figure 16A: γH2AX signal at the sex body of normal cells and all washout cells but all over the chromatin in ~80% of centrinone treated cells.

B Wild type (normal) + centrinone (abnormal)



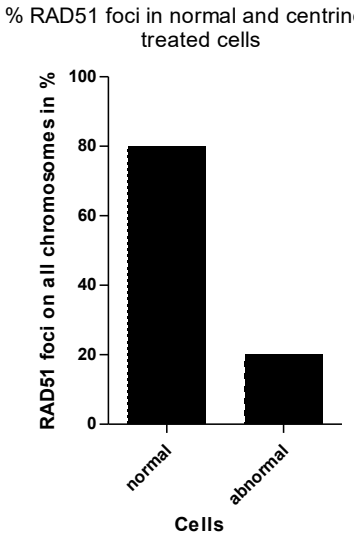


Figure 16B: RAD51 foci at the sex body of normal cells but all over the chromosomes of cenntrinone treated cells (pachytene and diplotene stages) N=200 per experiment and each experiment was Done at least thrice, scale bars: 10µm

PRELIMINARY DATA FROM ENU MICE

Harris et al., 2011 carried out a genome-wide mutagenesis using N-ethyl-N-nitrosurea (ENU) in male mice to identify genes potentially involved in spermatogenesis and male infertility. ENU introduces single nucleotide mutations which typically result in missense mutations (Justice et al., 1999), the resulting mutagenized mice identified 12 candidate genes using a SNP panel. On further sequencing of the exons and exon-intron boundaries, no mutations were identified except in *Plk4*.The amino terminal Serine/threonine kinase domain of *Plk4* (residues 12-265) in mice and man, contains the catalytic active site of the protein. The authors identified a novel heterozygous missense mutation in this domain of *Plk4*, altering an isoleucine to asparagine at residue 242 (I242N) (**Figure 17**). The homozygous *Plk4*$^{I242N/I242N}$ mutation is embryonically lethal and so all analyses were performed using the heterozygous *Plk4*$^{+/I242N}$ mice. Heterozygous *Plk4*$^{+/I242N}$ mice showed a reduction in testis to body weight ratio with affected animals reduced by 17.5% compared with the wild type littermates at 6 weeks of age (**Figure 18**). Testis histology was normal at postnatal day 1 (P1) but germ cell loss was detected at P10 and subsequently. The defective tubules were identified to contain only sertoli cells.

Figure 17: Schematic of the functional domains of PLK4 and identification of the ENU-induced *Plk4* I242N mutation (Harris et al., 2011)

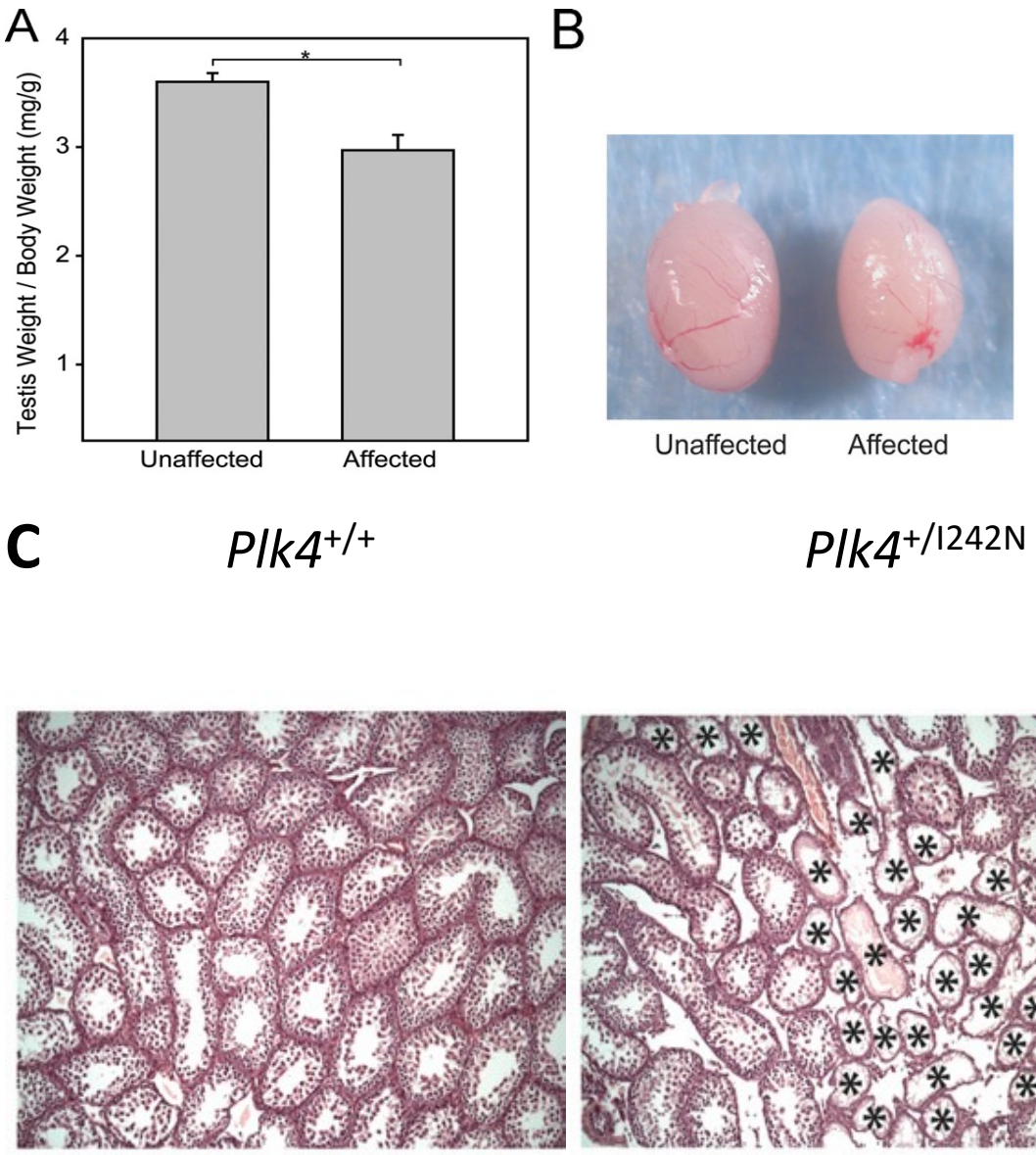
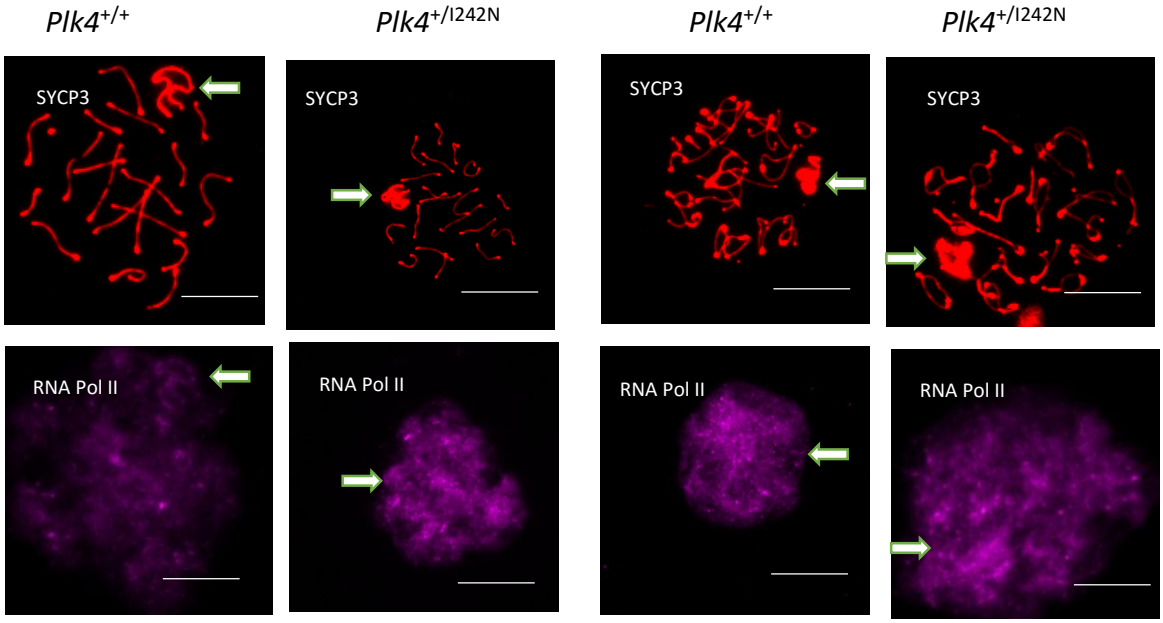


Figure 18: Phenotypic features of ENU mutant. **(A)** Quantification of testis weights at 6 weeks of age **(B)** Gross examination of whole testes at 6 weeks of age **(C)** Histological sections of wild type (*Plk4*$^{+/+}$) and mutant (*Plk4*$^{+/I242N}$) at postnatal day 21. Asterisks in *Plk4*$^{+/I242N}$ denote seminiferous tubules devoid of germ cells (Harris et al., 2011)

PROPHASE I DEFECTS IN ENU MICE

We assessed the localization of RNA polymerase II (RNA pol II) in wild type and ENU mice. RNA pol II is a marker of transcriptional activity which is normally absent at the sex body during pachynema and diplonema to indicate transcriptional silencing in wild type mice. The heterozygous *Plk4*$^{+/I242N}$ mutant fails to completely exclude RNA pol II at the sex body suggesting that PLK4 influences transcriptional activity and therefore meiotic silencing. The quantification of the fluorescence intensity of the RNA pol II signal at the sex body compared to the total chromatin in both wild type and mutant showed a statistically significant difference (**Figure 19**)

A



B

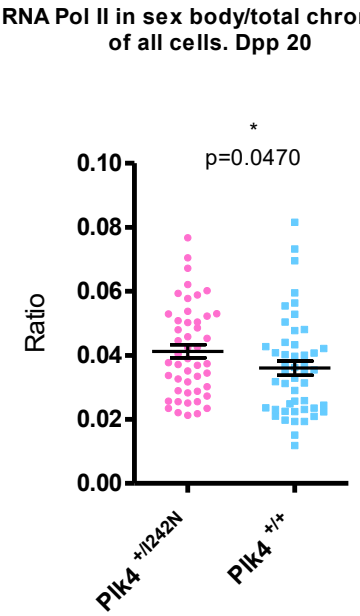
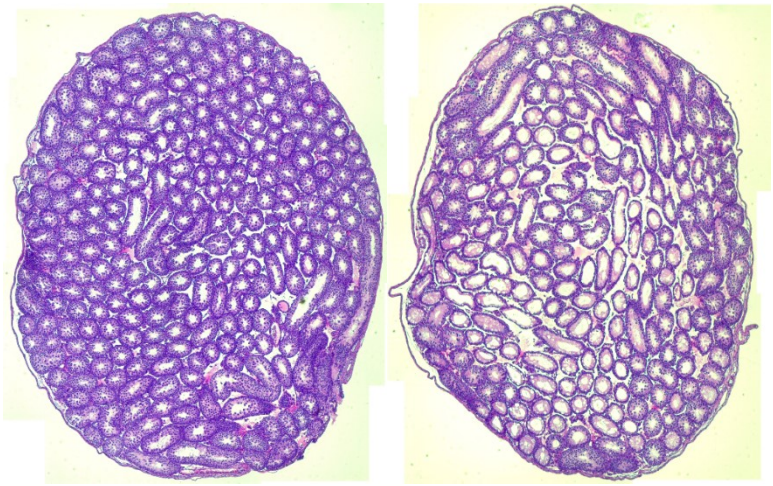


Figure 19: **A**) RNA Pol II localization in wild type (*Plk4*$^{+/+}$) and mutant (*Plk4*$^{+/I242N}$) shown for pachytene and diplotene stage cells, white arrowheads point to the position of the sex body. Scale bars: 10µm **B)** quantification shown below, N=48 for each genotype, error bars represent mean + SEM, Statistical analysis was done using Mann-Whitney test showing slight significance (*) with P = 0.0470.

HISTOLOGICAL DEFECTS IN ENU MICE

We also assessed the seminiferous tubules from wild type (*Plk4*$^{+/+}$) and ENU heterozygous mutant (*Plk4*$^{+/I242N}$) from 14 dpp to 20 dpp to examine for defects and germ cell loss as observed by Harris et al., 2012. As cells progress through spermatogenesis, the seminiferous tubules are lined by a complex stratified epithelium containing two distinct populations of cells- spermatogenic cells which will eventually form spermatozoa and sertoli cells which are the epithelial cells that surround the germ cells providing nutrients for them as well as phagocytosing excess spermatid cytoplasm. Spermatogonia are the first set of spermatogenic cells always located in contact with the basal lamina of the seminiferous tubules, they give rise to primary spermatocytes which undergo the first meiotic division and secondary spermatocytes which undergo the second meiotic division, these eventually form spermatids seen in the luminal part of the tubules. ENU heterozygous mutants (*Plk4*$^{+/I242N}$) clearly show defective tubules as compared to the corresponding wild type (*Plk4*$^{+/+}$) of each stage. Shown below at various magnifications is the cross section of whole testis (dpp 16) of wild type (*Plk4*$^{+/+}$) which is seen as very compact with spermatogonia and primary spermatocytes. In contrast, the mutant (*Plk4*$^{+/I242N}$) cross sections display loss of primary spermatocytes (**Figure 20**). This is followed by quantification of the number of cells in seminiferous tubules of wild type (*Plk4*$^{+/+}$) and mutant (*Plk4*$^{+/I242N}$), the area of the seminiferous tubules of wild type (*Plk4*$^{+/+}$) and mutant (*Plk4*$^{+/I242N}$) and the ratio of the number of cells per tubule to the area of the tubule for wild type (*Plk4*$^{+/+}$) and mutant (*Plk4*$^{+/I242N}$). Each quantification shows a significant difference for the wild type versus the mutant.

A *Plk4*$^{+/+}$ *Plk4*$^{+/I242N}$



B *Plk4*$^{+/+}$ *Plk4*$^{+/I242}$

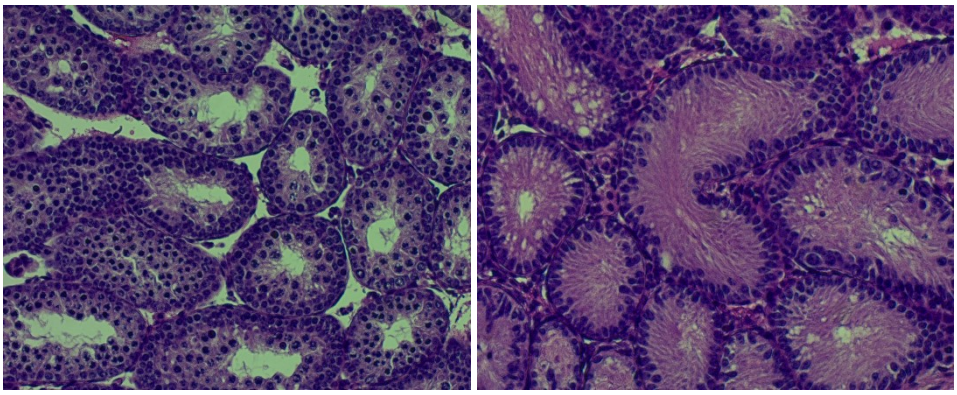
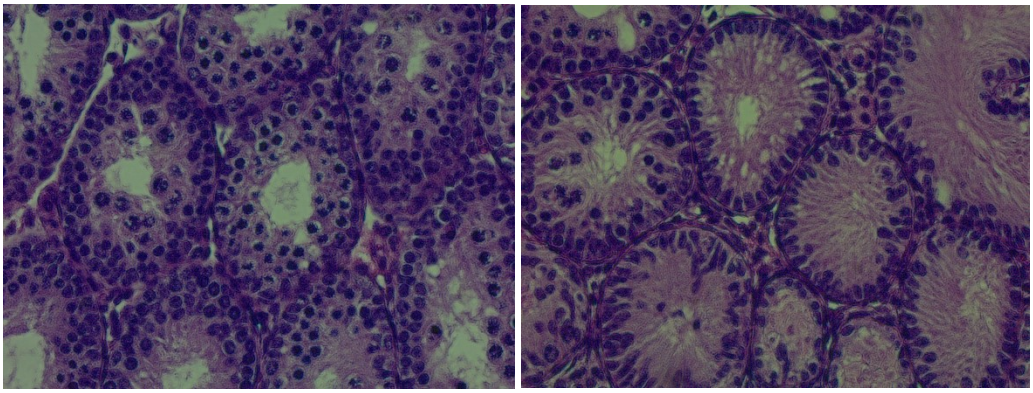


Figure 20: Histologic cross section of whole testis of wild type (*Plk4*$^{+/+}$) and mutant (*Plk4*$^{+/I242N}$) - **A**) x10 magnification **B**) x20 magnification

C *Plk4*$^{+/+}$ *Plk4*$^{+/I242}$



D

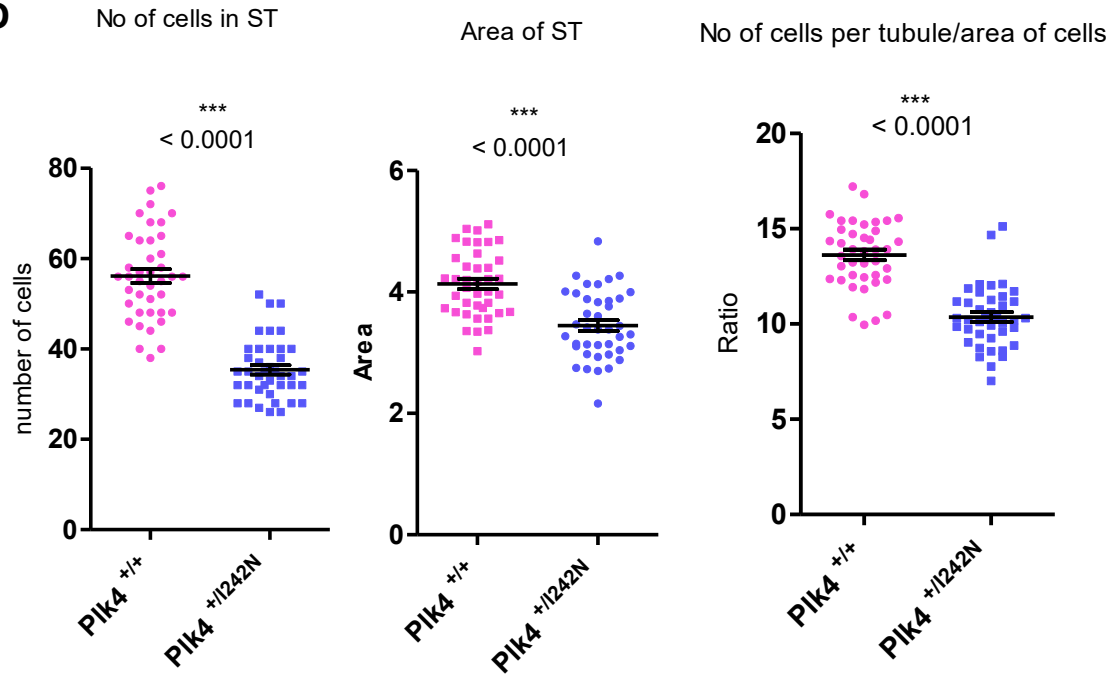


Figure 20 cont: Histologic cross section of whole testis of wild type (*Plk4*$^{+/+}$) and mutant (*Plk4*$^{+/I242N}$) - **C**) x40 magnification **D**) various quantification measures of seminiferous tubules in wild type (*Plk4*$^{+/+}$) and mutant (*Plk4*$^{+/I242N}$). N=40 for each genotype, error bars represent mean + SEM, Statistical analysis was done using Mann-Whitney test showing extreme significance (***) with P < 0.0001 in each case.

DISCUSSION

So far the role of PLK4 has been established in centriole biogenesis. However, a mutation in its kinase domain resulting in hypogonadism and patchy germ cell loss (Harris et al., 2011) as well as previous findings in our lab provide the premise for a novel function for PLK4 during spermatogenesis. The goal of this study was to further our knowledge on the role of the protein PLK4 in spermatogenesis by assessing the progression of meiosis while inhibiting its action and also in the heterozygous *Plk4*$^{+/I242N}$ mutant. In addition to polo-like kinases, other kinases such as cyclin-dependent kinases (CDKs) and Aurora kinases as well as phosphatases are key regulators of cell cycle events. Previous studies have successfully used small molecule inhibitor analyses to indicate their various roles during spermatogenesis.

The prophase to metaphase I (G2/MI) transition can be artificially stimulated by addition of the phosphatase inhibitor, okadaic acid (OA) (Wiltshire et al., 1995). The signature event of the G2/MI transition is the disassembly of the SC followed by chromatin condensation and compaction of the MI bivalent chromosomes. Sun and Handel, 2008 studied the enzymatic control of these events using the small molecule inhibitors- butyrolactone I (BLI), an inhibitor of cyclin dependent kinases (CDKs) and ZM447439 (ZM), an inhibitor of Aurora kinases and show that neither BLI nor ZM inhibited disassembly of the central element of the SC. The central element of the SC, SYCP1 bridges the two lateral elements of the SC and is essential for synapsis as synapsis does not occur in mice lacking SYCP1 (de Vries et al., 2005). The SC disassembles as

meiotic cells progress from pachynema through diplonema. The hallmark of this exit is desynapsis and it is marked by the removal of the central element of the SC, SYCP1 (Moens, 1995). G2/MI transition is predominantly controlled by the metaphase promoting factor (MPF) which consist of cyclin subunits as well as other candidate kinases including the Aurora kinases (Carmena and Earnshaw, 2003). Taking advantage of the experimental induction of the G2/MI transition with OA, together with the addition of the small molecule inhibitors, Sun and Handel, 2008 show that desynapsis and removal of SYCP1 began rapidly after treatment with OA or OA + BLI. Thus desynapsis is not sensitive to the CDK inhibitor BLI. Also, the OA-induced disassembly of SYCP1 was not affected by ZM as spermatocytes treated with either OA or OA + ZM completed desynapsis within 3 hours. The authors therefore use inhibitor analysis to reveal that CDKs and Aurora kinases are not involved in the disassembly of the SC central element. In a subsequent inhibitor analysis study using the potent and small molecule inhibitor of mammalian PLK1, BI 2536 optimized by Steegmaier et al., 2007 and OA to also induce G2/MI transition, Jordan et al., 2012 show that inhibiting PLK1 inhibited the OA –induced dissassebly of the SC central and lateral elements.

Cyclin-dependent kinase 2 (CDK2) is known to play important roles in mammalian cell cycle progression, both at the transition from G1 to S and through S phase. *Cdk2*$^{-/-}$ spermatocytes show incomplete chromosome pairing and formation of sex body, an extensive non-homologous synapsis and an arrest at pachytene-like stage with unrepaired programmed double-strand breaks (Viera et al., 2009). Phosphorylation of CDK2 at threonine 160 (p-CDK2) is essential for its kinase activity and has been

implicated as a potential regulator for MSCI in male mice (Wang et al., 2014). Mevastatin, an inhibitor of cholesterol synthesis, inhibits cell growth by inhibiting CDK2 (Ukomadu and Dutta, 2003). Wang et al., (2014) treated pachytene stage spermatocytes with mevastatin and they report an absence of p-CDK2 at the sex body which is normally not the case in non-inhibited cells. They also notice an upregulation of sex chromosome genes as well as RNA Polymerase II staining visible on the sex chromosome in mevastatin-treated spermatocytes therefore suggesting a role for p-CDK2 in the transcriptional activity of sex chromosome genes and also in MSCI. Inhibition of CDK2 has also been shown to block centrosome duplication (Matsumoto et al., 1999; Lacey et al., 1999). Overexpression of PLK4 cannot cause centrosome amplification in the absence of CDK2 activity, conversely, CDK2 activity cannot cause centrosome amplification in the absence of PLK4 activity clearly indicating that both proteins cooperate to control centrosome duplication (Habedanck et al., 2005).

The data reported here confirm the localization of PLK4 to the sex body during pachynema and diplonema of meiotic prophase I. Colocalization of PLK4 with γH2AX during pachytene suggest that PLK4 possesses a novel role in DNA repair and MSCI. By treating the primary spermatocytes of wild type mice with centrinone, a PLK4 inhibitor, we observe delayed meiotic progression but not meiotic arrest. We also observe a widespread localization of γH2AX throughout the chromatin of centrinone treated cells. This phenotype suggests either delayed DDR or the presence of new DNA damage and is confirmed by the presence of RAD51 foci on the chromosomes of pachytene stage cells. To rule out the possibility of the DNA damage resulting from toxicity of centrinone

rather than PLK4 inhibition, washout experiments were carried out and the retrieved cells showed phenotype similar to the wild type cells confirming the inhibition was causative for the observed phenotype. However, because γH2AX may also be a signal for apoptosis in cells, use of other apoptosis markers such as cleaved caspase-3 or cleaved poly (ADP) ribose polymerase will be necessary to confirm if the cells are dying in response to centrinone treatment.

Due to the embryonic lethality of the homozygous *Plk4*$^{I242N/I242N}$ mice, the I242N mutation can only be studied in the heterozygous state. This mutation in PLK4 results in a change from isoleucine to asparagine at amino acid 242, because isoleucine is an aliphatic, hydrophobic amino acid whereas asparagine is polar and hydrophilic, it is likely that this substitution results in a conformational change in the kinase domain of PLK4 (Harris et al., 2011). PLK4 mutation in other organisms has been studied. *Drosophila melanogaster* PLK4 mutants lose their centrioles during the mitotic divisions preceding male meiosis but still produce the same number of primary spermatocyte cysts as the wild type, the spermatids however lack centrioles and are unable to produce axonemes (bundle of microtubules that form the core of the sperm flagellum and are responsible for movement) (Bettencourt-Dias et al., 2005). When the C terminus of the PLK4 homolog in *Caenorhabditis elegans* is truncated, there is decreased mitotic centriole duplication and centrosome amplification in the meiotic cycle (Peters et al., 2010). Interestingly, various studies have reported cross talks between the centrosomes, DNA repair and infertility. DNA damage response and repair proteins such as BRCA1 and ATM/ATR kinases localize to the centrosome, ATR is one of the proteins responsible for

Seckel syndrome, an autosomal recessive disorder characterized by intrauterine growth retardation, severe proportionate short stature and microcephaly (Rauch et al., 2008). ATR is also known to be involved in DNA damage response and its knockdown by siRNA leads to centrosome overduplication (Collis et al., 2008). BRCA1 is also involved in DNA repair and its knockdown leads to centrosome overduplication as well (Ko et al., 2006). The dysfunction of these proteins are known to cause tumorigenesis due to defective maintenance of centrosomes as well as impaired DNA repair (Shimada and Komatsu, 2009). In another study investigating the contribution of the PBD of Cdc5 (yeast polo-like kinase) in DNA damage response, the authors show that the PBD of Cdc5 is essential to mediate adaptation to persistent DNA damage and acts by targeting Cdc5 kinase activity to the yeast centrosome (Ratsima et al., 2016). Furthermore, the centrosomal protein CEP63 has been identified as a target for DDR kinases ATM and ATR in mitosis (Smith et al., 2009). *Cep63* deficient cells and tissues do not show obvious defects in DNA damage signaling but exhibit impaired centriole duplication. Moreover, *Cep63* deficient mice are infertile exhibiting severe defects in meiotic recombination and a complete block in the generation of sperm (Marjanovic et al., 2015). The authors use SYCP3 and SYCP1 markers and show increased leptotene and zygotene stage cells in the mutant compared to the wild type, similar numbers of pachytene cells but very few cells (4%) progressed to diplotene stage suggesting progressive cell loss during prophase I. There was also increased number of RAD 51 and DMC1 foci observed from leptotene to zygotene in *Cep63* mutants indicating that the formation of DSBs was not defective, however, their repair was defective as many pachytene and diplotene stage cells

exhibited diffuse γH2AX staining not confined to the sex body consistent with delayed repair. The mechanisms underlying the contribution of polo-like kinases to DNA damage response-particularly to the adaptation to persistent DNA damage still remain elusive (Archambault et al., 2015).

We assessed the histology of seminiferous tubules from wild type and heterozygous *Plk4*$^{+/I242N}$ mutant from 14-18 dpp. While the wild type tubules are tightly packed with germ cells, the *Plk4*$^{+/I242N}$ mutant appears morphologically abnormal with most tubules exhibiting more spacious lumen as well as loss of germ cells. Furthermore, RNA Polymerase II signal was not robustly excluded on the sex chromosomes of *Plk4*$^{+/I242N}$ as seen in wild type, suggesting ongoing transcription at the sex body which can be confirmed by real-time quantitative PCR and assessment of sex-linked genes for upregulated activity. Taken together, these data provide evidence for the role of PLK4 in spermatogenesis and more specifically, we have shown evidence that PLK4 is required for efficient DNA damage response and repair processes that are essential for meiotic progression. Our future work will define the mechanistic roles of PLK4 both within the nucleus and at the centromeres during spermatogenesis. Based on recent evidence, it is likely that these roles are inter-dependent.

In summary, various studies have shown an increasing number of proteins being found to localize at the sex body and taking part in the process of MSCI, their functions and mechanistic details are yet to be fully elucidated. We propose PLK4 as another major player and regulator of MSCI in male mice, further studies are however required to completely elucidate its involvement and mechanism. Future directions to fully

understand the role of PLK4 in spermatogenesis include: studying the mechanism of PLK4 recruitment to the chromosome axes and its possible substrates, further assessment of DNA damage/repair in the absence of PLK4, assessment of centriole function while inhibiting PLK4, assessment of the expression of sex-linked genes in wild type versus *Plk4* mutant primary spermatocytes, and finally generating a conditional knockout model to assess the effect of a homozygous *Plk4* mutation.

PUBLIC HEALTH SIGNIFICANCE

Polo-like kinase 4 (PLK4) is known to be the master regulator of centriole biogenesis. Centriole duplication is tightly controlled in the cell such that each cell has precisely two centrosomes, supernumerary centrosomes have been postulated to contribute to tumorigenesis and are prevalent in cancer (Firat-karalar and Stearns, 2014; Godinho and Pellman, 2014). Previous studies established that knockdown of the tumour suppressor p53 led to centrosome amplification in mouse fibroblasts and skin tumours (Fukasawa, 2007), further studies have also established that centrosome abnormalities are common in a variety of human tumours such as breast, prostate, lung, cancer and the brain (Lingle et al., 1998; Pihan et al., 1998).

Various gene mutations including overexpression of *Plk4* have also been linked with primary recessive microcephaly (MCPH), a neuro-developmental disorder characterized by a reduction in brain size during fetal development (Marthiens et al., 2013). Most of the causative genes for microcephaly are linked to disruption in centrosome integrity. In a short medical report following the imaging and laboratory phenotyping of a consanguineous Saudi Arabian family in which two full siblings and one half sibling presented with classical features of Seckel syndrome, the phenotype was mapped to a single novel locus in which they identified a five base pair deletion in *Plk4* with schematic shown below (Shaheen et al., 2014) (**Figure 21**). Recent evidence also suggests a complex interaction between PLK4 and CEP152 centrosomal protein to form a scaffold upon which mature centrioles form and in the absence of PLK4, this step fails

(Kim et al., 2013). *Cep152* has also been shown to be mutated in patients with Seckel syndrome (Kalay et al., 2011). Furthermore, CEP152 interacts with another centrosomal protein, CEP63 and are dependent on one another for centrosomal localization (Brown et al., 2013). Mice lacking the expression of *Cep63* recapitulates the pathological outcomes reported in human patients with *Cep63* mutations including growth defects and microcephaly (Sir et al., 2011; Marjanovic et al., 2015). Having established the role of PLK4 in the centrosomes and the various diseases associated, also relevant is its role in spermatogenesis and fertility.

Infertility has been classified as a disease by the American Society for Reproductive Medicine, stating also that the male partner is usually the sole cause or a contributor in 40% of cases. World Health Organization reports that one in every four couples in developing countries is affected and on a global scale, infertility is said to affect 15% of couples amounting to 48.5 million couples with males being solely responsible for 20-30% of these cases (Agarwal et al., 2015). Errors in chromosomal segregation during meiosis is associated with aneuploidy. Aneuploidy is the condition of having less than (monosomy) or more than (polysomy) the normal diploid number of chromosomes. Klinefelter syndrome (47 XXY) for example, is the most pervasive sex chromosomal anomaly affecting approximately 1:600 males and leading to hypogonadism and infertility (Nielsen and Wohlert, 1990). Turner syndrome (45, X) is also another frequent chromosomal abnormality caused by a missing or incomplete X chromosome. It is found in more than 7% of all spontaneous abortions and only 2% of the embryos survive to term. People with Turner syndrome develop as females and it is

typically caused by non-disjunction of the sex chromosomes during the formation of a sperm (or egg). 80% of Turner syndrome cases are the result of paternal non-disjunction resulting when sperm with no X chromosome unites with an egg to form an embryo with just one chromosome rather than two (Martinez-Pasarell et al., 1999; Tissot and Kaufman, Human Genetics).

Harris et al., 2011 while trying to identify candidate genes potentially involved in spermatogenesis and male fertility carried out a genome-wide mutagenesis in mice and discovered *Plk4* as a novel gene implicated in hypogonadism and patchy germ cell loss. Male mice with point mutation in the *Plk4* gene show azoospermia associated with germ cell loss, this phenotype was also complemented by researchers who carried out mutational analysis of human patients with azoospermia (Miyamoto et al., 2015). Our results and findings implicate a role for PLK4 in MSCI, the process of transcriptional inactivation of homologs that fail to synapse during meiosis and in this case the heterologous X and Y chromosome in the male germ cell. This process of MSCI is thought to act as a meiotic surveillance mechanism to prevent the generation of aneuploid gametes and eliminate defective meiotic cells (Nagaoka et al., 2012). Various studies have shown pachytene stage arrest and apoptosis in mouse mutants where MSCI fails indicating the process as essential for male meiosis and failure to properly execute this mechanism is associated with meiotic arrest and eventually causes male infertility (Royo et al., 2010). In conclusion, a better understanding of the function of PLK4 and other proteins involved in MSCI, their mechanism and action, would lead to

advances and solutions to infertility, reduce incidences of sex chromosome aneuploidy and improve reproductive health in general.

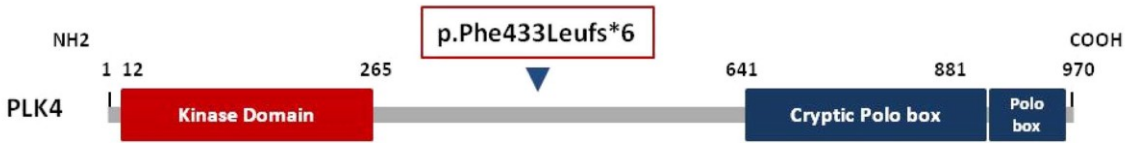


Figure 21: Schematic of *Plk4* showing the site of mutation in Seckel syndrome patients

REFERENCES

Abe, S., Nagasaka, K., Hirayama, Y., Kozuka-Hata, H., Oyama, M., Aoyagi, Y., et al. (2011). The initial phase of chromosome condensation requires Cdk1-mediated phosphorylation of the CAP-D3 subunit of condensin II. *Genes & Development, 25*(8), 863-874.

Agarwal, A., Mulgund, A., Hamada, A., & Chyatte, M. R. (2015). A unique view on male infertility around the globe. Reproductive Biology and Endocrinology: RB&E, 13, 37.

American Society for Reproductive Medicine. (2016). Quick facts about fertility. Retrieved April, 2016, 2016, from https://www.asrm.org/detail.aspx?id=2322

Archambault, V., Lepine, G., & Kachaner, D. (2015). Understanding the polo kinase machine. Oncogene, 34(37), 4799-4807.

Archambault, V., & Glover, D. M. (2009). Polo-like kinases: Conservation and divergence in their functions and regulation. Nature Reviews. Molecular Cell Biology, 10(4), 265-275.

Barr, F. A., Elliott, P. R., & Gruneberg, U. (2011). Protein phosphatases and the regulation of mitosis. Journal of Cell Science, 124(14), 2323-2334. doi:10.1242/jcs.087106

Bettencourt-Dias, M., Rodrigues-Martins, A., Carpenter, L., Riparbelli, M., Lehmann, L., Gatt, M. K., et al. (2005). SAK/PLK4 is required for centriole duplication and flagella development. Current Biology, 15(24), 2199-2207.

Brown NJ, Marjanovic M, Luders J, Stracker TH, Costanzo V. (2013). Cep63 and Cep152 cooperate to ensure centriole duplication. PLoS ONE 8(7): E69986. Doi:10.1371/journal.Pone.0069986

Brownlee, C. W., Klebba, J. E., Buster, D. W., & Rogers, G. C. (2011). The protein phosphatase 2A regulatory subunit twins stabilizes Plk4 to induce centriole amplification. The Journal of Cell Biology, 195(2), 231-243.

Burgoyne, P. S., Mahadevaiah, S. K., & Turner, J. M. A. (2009). The consequences of asynapsis for mammalian meiosis. Nature Reviews. Genetics, 10(3), 207-216.

Caenepeel, S., Charydczak, G., Sudarsanam, S., Hunter, T., & Manning, G. (2004). The mouse kinome: Discovery and comparative genomics of all mouse protein kinases. Proceedings of the National Academy of Sciences of the United States of America, 101(32), 11707-11712.

Carmena, M., & Earnshaw, W. C. (2003). The cellular geography of aurora kinases. Nature Reviews. Molecular Cell Biology, 4(11), 842-854.

Carvalho-Santos, Z., Machado, P., Branco, P., Tavares-Cadete, F., Rodrigues-Martins, A., Pereira-Leal, J. B., et al. (2010). Stepwise evolution of the centriole-assembly pathway doi:10.1242/jcs.064931.

Collis, S. J., Ciccia, A., Deans, A. J., Hoaejaja, Z., Martin, J. S., Maslen, S. L., et al. (2008). FANCM and FAAP24 function in ATR-mediated checkpoint signaling independently of the fanconi anemia core complex. Molecular Cell, 32(3), 313-324.

Cunha-Ferreira, I., Bento, I., Pimenta-Marques, A., Jana, S. C., Lince-Faria, M., Duarte, P., et al. (2013). Regulation of autophosphorylation controls PLK4 self-destruction and centriole number. Current Biology, 23(22), 2245-2254.

de Carcer, G., Escobar, B., Higuero, A. M., Garcia, L., Anson, A., Perez, G., et al. (2011). Plk5, a polo box domain-only protein with specific roles in neuron differentiation and glioblastoma suppression. Molecular and Cellular Biology, 31(6), 1225-1239.

de Vries, F. A. T., de Boer, E., van den Bosch, M., Baarends, W. M., Ooms, M., Yuan, L., et al. (2005). Mouse Sycp1 functions in synaptonemal complex assembly, meiotic recombination, and XY body formation. Genes & Development, 19(11), 1376-1389.

Elia, A. E. H., Rellos, P., Haire, L. F., Chao, J. W., Ivins, F. J., Hoepker, K., et al. The molecular basis for phosphodependent substrate targeting and regulation of plks by the polo-box domain. Cell, 115(1), 83-95.

Firat-Karalar, E. N., & Stearns, T. (2014). The centriole duplication cycle. Philosophical Transactions of the Royal Society B: Biological Sciences, 369(1650), 20130460.

Fode, C., Binkert, C., & Dennis, J. W. (1996). Constitutive expression of murine sak-a suppresses cell growth and induces multinucleation. Molecular and Cellular Biology, 16(9), 4665-4672

Fukasawa, K. (2007). Oncogenes and tumour suppressors take on centrosomes. Nat Rev Cancer, 7(12), 911-924.

Girdler, F., Sessa, F., Patercoli, S., Villa, F., Musacchio, A., & Taylor, S. (2008). Molecular basis of drug resistance in aurora kinases. Chemistry & Biology, 15(6), 552-562.

Godinho, S. A., & Pellman, D. (2014). Causes and consequences of centrosome abnormalities in cancer. Philosophical Transactions of the Royal Society B: Biological Sciences, 369(1650), 20130467.

Habedanck, R., Stierhof, Y., Wilkinson, C. J., & Nigg, E. A. (2005). The polo kinase Plk4 functions in centriole duplication. Nature Cell Biology, 7(11), 1140-1146.

Handel, M. A. (2004). The XY body: A specialized meiotic chromatin domain. Experimental Cell Research, 296(1), 57-63.

Handel, M. A., & Schimenti, J. C. (2010). Genetics of mammalian meiosis: Regulation, dynamics and impact on fertility. Nature Reviews. Genetics, 11(2), 124-136.

Harris, R. M., Weiss, J., & Jameson, J. L. (2011). Male hypogonadism and germ cell loss caused by a mutation in polo-like kinase 4. Endocrinology, 152(10), 3975-3985.

Harton, G. L., & Tempest, H. G. (2011). Chromosomal disorders and male infertility. Asian Journal of Andrology, 14(1), 32-39.

Heard, E., & Turner, J. (2011). Function of the sex chromosomes in mammalian fertility. Cold Spring Harbor Perspectives in Biology, 3(10)

Holland, A. J., Lan, W., & Cleveland, D. W. (2010). Centriole duplication: A lesson in self-control. Cell Cycle, 9(14), 2731-2736.

Hudson, J. W., Kozarova, A., Cheung, P., Macmillan, J. C., Swallow, C. J., Cross, J. C., et al. (2001). Late mitotic failure in mice lacking sak, a polo-like kinase. Current Biology, 11(6), 441-446.

Ines Cunha-Ferreira, Ines Bento and Mónica Bettencourt-Dias. (2009). From zero to many: Control of centriole number in development and disease. Doi: 10.1111/j.1600-0854.2009.00905.x, Volume 10(Issue 5)

James E Sillibourne and Michel Bornens. (2010). Polo-like kinase 4: The odd one out of the family. Cell Division20105:25(DOI: 10.1186/1747-1028-5-25)

Jana, S., Bazan, J. & Dias, M. (2012). Polo boxes come out of the crypt: A new view of PLK function and evolution. Structure, 20(11), 1801-1804.

Jeffrey Weiss , Lisa A. Hurley, Rebecca M. Harris, Courtney Finlayson, Minghan Tong, Lisa A. Fisher, Jennifer L. Moran, David R. Beier, Christopher Mason, J. Larry Jameson. (2012). ENU mutagenesis in mice identifies candidate genes for hypogonadism. Doi: 10.1007/s00335-011-9388-5, 23(5)

Jordan, P. W., Karppinen, J., & Handel, M. A. (2012). Polo-like kinase is required for synaptonemal complex disassembly and phosphorylation in mouse spermatocytes doi:10.1242/jcs.105015

Justice, M. J., Noveroske, J. K., Weber, J. S., Zheng, B., & Bradley, A. (1999). Mouse ENU mutagenesis. Human Molecular Genetics, 8(10), 1955-1963.

Kalay, E., Yigit, G., Aslan, Y., Brown, K. E., Pohl, E., Bicknell, L. S., et al. (2011). CEP152 is a genome maintenance protein disrupted in Seckel syndrome. Nature Genetics, 43(1), 23-26.

Karaman, M. W., Herrgard, S., Treiber, D. K., Gallant, P., Atteridge, C. E., Campbell, B. T., et al. (2008). A quantitative analysis of kinase inhibitor selectivity. Nat Biotech, 26(1), 127-132.

Kim, T., Park, J., Shukla, A., Choi, S., Murugan, R. N., Lee, J. H., et al. (2013). Hierarchical recruitment of Plk4 and regulation of centriole biogenesis by two centrosomal scaffolds, Cep192 and Cep152. Proceedings of the National Academy of Sciences, 110(50), E4849-E4857.

Klebba, J., Buster, D., Nguyen, A., Swatkoski, S., Gucek, M., Rusan, N., et al. (2013). Polo-like kinase 4 autodestructs by generating its slimb-binding phosphodegron. Current Biology, 23(22), 2255-2261.

Ko, M. J., Murata, K., Hwang, D., & Parvin, J. D. (2005). Inhibition of BRCA1 in breast cell lines causes the centrosome duplication cycle to be disconnected from the cell cycle. Oncogene, 25(2), 298-303.

Kothe, M., Kohls, D., Low, S., Coli, R., Cheng, A. C., Jacques, S. L., et al. (2007). Structure of the catalytic domain of human polo-like kinase 1, Biochemistry, 46(20), 5960-5971.

Lacey, K. R., Jackson, P. K., & Stearns, T. (1999). Cyclin-dependent kinase control of centrosome duplication. Proceedings of the National Academy of Sciences, 96(6), 2817-2822.

Ledoux, A. C., Sellier, H., Gillies, K., Iannetti, A., James, J., & Perkins, N. D. (2013). NFkB regulates expression of polo-like kinase 4. Cell Cycle, 12(18), 3052-3062.

Leung, G. C., Hudson, J. W., Kozarova, A., Davidson, A., Dennis, J. W., & Sicheri, F. (2002). The sak polo-box comprises a structural domain sufficient for mitotic subcellular localization. Nat Struct Mol Biol, 9(10), 719-724.

Lingle, W. L., Lutz, W. H., Ingle, J. N., Maihle, N. J., & Salisbury, J. L. (1998). Centrosome hypertrophy in human breast tumors: Implications for genomic stability and cell polarity. Proceedings of the National Academy of Sciences, 95(6), 2950-2955.

Ma, S., Charron, J., & Erikson, R. L. (2003). Role of Plk2 (snk) in mouse development and cell proliferation. Molecular and Cellular Biology, 23(19), 6936-6943.

Mahadevaiah, S. K., Evans, E. P., & Burgoyne, P. S. (2000). An analysis of meiotic impairment and of sex chromosome associations throughout meiosis in XYY mice.

Mahadevaiah, S. K., Turner, J. M. A., Baudat, F., Rogakou, E. P., de Boer, P., Blanco-Rodriguez, J., et al. (2001). Recombinational DNA double-strand breaks in mice precede synapsis. Nature Genetics, 27(3), 271-276.

Marjanovic, M., Sanchez-Huertas, C., Terre B., Gomez, R., Scheel, J. F., Pacheco, S., et al. (2015). CEP63 deficiency promotes p53-dependent microcephaly and reveals a role for the centrosome in meiotic recombination. Nature Communications, 6, 7676-7676.

Marthiens, V., Rujano, M. A., Pennetier, C., Tessier, S., Paul-Gilloteaux, P., & Basto, R. (2013). Centrosome amplification causes microcephaly. Nature Cell Biology, 15(7), 731-740.

Martinez-Pasarell, O., Templado, C., Vicens-Calvet, E., Egozcue, J., & Nogues, C. (1999). Paternal sex chromosome aneuploidy as a possible origin of turner syndrome in monozygotic twins: Case report. Human Reproduction, 14(11), 2735-2738.

Matsumoto, Y., Hayashi, K., & Nishida, E. (1999). Cyclin-dependent kinase 2 (Cdk2) is required for centrosome duplication in mammalian cells. Current Biology, 9(8), 429-432.

Miyamoto, T., Bando, Y., Koh, E., Tsujimura, A., Miyagawa, Y., Iijima, M., et al. (2015). A PLK4 mutation causing azoospermia in a man with sertoli cell-only syndrome. Andrology, 4(1), 75-81.

Nagaoka, S. I., Hassold, T. J., & Hunt, P. A. (2012). Human aneuploidy: Mechanisms and new insights into an age-old problem. Nature Reviews. Genetics, 13(7), 493-504.

Nielsen J1, W. M. (1990). Sex chromosome abnormalities found among 34,910 newborn children: Results from a 13-year incidence study in arhus, denmark**.** Birth Defects Orig Artic Ser.;26(4):209-23.,

Peel, N., Dougherty, M., Goeres, J., Liu, Y., & O'Connell, K. F. (2012). The C. elegans F-box proteins LIN-23 and SEL-10 antagonize centrosome duplication by regulating ZYG-1 levels doi:10.1242/jcs.097105

Peters, N., Perez, D. E., Song, M. H., Liu, Y., Muller-Reichert, T., Caron, C., et al. (2010). Control of mitotic and meiotic centriole duplication by the Plk4-related kinase ZYG-1 doi:10.1242/jcs.050682.

Pihan, G. A., Purohit, A., Wallace, J., Knecht, H., Woda, B., Quesenberry, P., et al. (1998). Centrosome defects and genetic instability in malignant tumors. Cancer Research, 58(17), 3974-3985.

Ratsima, H., Serrano, D., Pascariu, M., & Dae Amours, D. Centrosome-dependent bypass of the DNA damage checkpoint by the polo kinase Cdc5. Cell Reports, 14(6), 1422-1434.

Rauch, A., Thiel, C. T., Schindler, D., Wick, U., Crow, Y. J., Ekici, A. B., et al. (2008). Mutations in the pericentrin (PCNT) gene cause primordial dwarfism doi:10.1126/science.1151174

Rechsteiner, M., & Rogers, S. W. (1996). PEST sequences and regulation by proteolysis. Trends in Biochemical Sciences, 21(7), 267-271.

Robert Tissot and Elliot Kaufman. Human genetics- chromosomal inheritance, 2016, from https://www.uic.edu/classes/bms/bms655/lesson10.html

Royo, H., Polikiewicz, G., Mahadevaiah, S. K., Prosser, H., Mitchell, M., Bradley, A., et al. (2010). Evidence that meiotic sex chromosome inactivation is essential for male fertility. Current Biology, 20(23), 2117-2123.

Royo, H., Prosser, H., Ruzankina, Y., Mahadevaiah, S. K., Cloutier, J. M., Baumann, M., et al. (2013). ATR acts stage specifically to regulate multiple aspects of mammalian meiotic silencing. Genes & Development, 27(13), 1484-1494.

Shaheen, R., Al Tala, S., Almoisheer, A., & Alkuraya, F. S. (2014). Mutation in PLK4, encoding a master regulator of centriole formation, defines a novel locus for primordial dwarfism. Journal of Medical Genetics, 51(12), 814-816.

Shimada, M., & Komatsu, K. (2009). Emerging connection between centrosome and DNA repair machinery. Journal of Radiation Research, 50(4), 295-301.

Siepka, S. M., & Takahashi, J. S. (2005). Forward genetic screens to identify circadian rhythm mutants in mice. Methods in Enzymology, 393, 219-229.

Sillibourne, J. E., Tack, F., Vloemans, N., Boeckx, A., Thambirajah, S., Bonnet, P., et al. (2010). Autophosphorylation of polo-like kinase 4 and its role in centriole duplication. Molecular Biology of the Cell, 21(4), 547-561.

Sir, J., Barr, A. R., Nicholas, A. K., Carvalho, O. P., Khurshid, M., Sossick, A., et al. (2011). A primary microcephaly protein complex forms a ring around parental centrioles. Nature Genetics, 43(11), 1147-1153.

Slevin, L., Nye, J., Pinkerton, D., Buster, D., Rogers, G., & Slep, K. (2012). The structure of the Plk4 cryptic polo box reveals two tandem polo boxes required for centriole duplication. Structure, 20(11), 1905-1917.

Sloane, D. A., Trikic, M. Z., Chu, M. L. H., Lamers, M. B. A. C., Mason, C. S., Mueller, I., et al. (2010). Drug-resistant aurora A mutants for cellular target validation of the small molecule kinase inhibitors MLN8054 and MLN8237. ACS Chemical Biology, 5(6), 563-576.

Smith, E., Dejsuphong, D., Balestrini, A., Hampel, M., Lenz, C., Takeda, S., et al. (2009). An ATM- and ATR-dependent checkpoint inactivates spindle assembly by targeting CEP63. Nature Cell Biology, 11(3), 278-285.

Steegmaier, M., Hoffmann, M., Baum, A.,Lacnairt, P., Petronczki, M., Kriaiaik, M., et al. (2007). BI 2536, a potent and selective inhibitor of polo-like kinase 1, inhibits tumor growth in vivo. Current Biology, 17(4), 316-322.

Sunkel, C. E., & Glover, D. M. (1988). Polo, a mitotic mutant of drosophila displaying abnormal spindle poles. Journal of Cell Science, 89(1), 25-38.

Thomas, N. S., & Hassold, T. J. (2003). Aberrant recombination and the origin of klinefelter syndrome. Human Reproduction Update, 9(4), 309-317.

Turner, J. M. A., Mahadevaiah, S. K., Fernandez-Capetillo, O., Nussenzweig, A., Xu, X., Deng, C., et al. (2005). Silencing of unsynapsed meiotic chromosomes in the mouse. Nature Genetics, 37(1), 41-47.

Ukomadu, C., & Dutta, A. (2003). Inhibition of cdk2 activating phosphorylation by mevastatin. Journal of Biological Chemistry, 278(7), 4840-4846.

Viera, A., Rufas, J. S., Martínez, I., Barbero, J. L., Ortega, S., & Suja, J. A. (2009). CDK2 is required for proper homologous pairing, recombination and sex-body formation during male mouse meiosis doi:10.1242/jcs.046706

Wang, L., Liu, W., Zhao, W., Song, G., Wang, G., Wang, X., et al. (2014). Phosphorylation of CDK2 on threonine 160 influences silencing of sex chromosome during male meiosis. Biology of Reproduction, 90(6), 138.

Wang, W., Soni, R. K., Uryu, K., & Bryan Tsou, M. (2011). The conversion of centrioles to centrosomes: Essential coupling of duplication with segregation. The Journal of Cell Biology, 193(4), 727-739.

WHO. (2016). Global prevalence of infertility, infecundity and childlessness. Retrieved April 2016, 2016, from http://www.who.int/reproductivehealth/topics/infertility/burden/en/

Wiltshire, T., Park, C., Caldwell, K. A., & Handel, M. A. (1995). Induced premature G2/M-phase transition in pachytene spermatocytes includes events unique to meiosis. Developmental Biology, 169(2), 557-567.

Wong, Y. L., Anzola, J. V., Davis, R. L., Yoon, M., Motamedi, A., Kroll, A., et al. (2015). Reversible centriole depletion with an inhibitor of polo-like kinase 4 doi:10.1126/science.aaa5111

Yamashita, Y., Kajigaya, S., Yoshida, K., Ueno, S., Ota, J., Ohmine, K., et al. (2001). Sak serine-threonine kinase acts as an effector of tec tyrosine kinase. Journal of Biological Chemistry, 276(42), 39012-39020.

Zitouni, S., Nabais, C., Jana, S. C., Guerrero, A., & Bettencourt-Dias, M. (2014). Polo-like kinases: Structural variations lead to multiple functions. Nature Reviews. Molecular Cell Biology, 15(7), 433-452.

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PERSONAL DATA

Date of birth: October 5, 1986

Place of birth: Rivers State, Nigeria

Citizenship: Nigerian

EDUCATION

Master of Science (ScM) in Biochemistry and Molecular Biology May 2016
Johns Hopkins Bloomberg School of Public Health

- Thesis advisor : Philip Jordan
- Thesis title : The role of Polo-like Kinase 4 (PLK4) in chromosome segregation and genome maintenance during mammalian meiosis

Master of Health Science (MHS) in Biochemistry and Molecular Biology 2015
Johns Hopkins Bloomberg School of Public Health

- Thesis advisor : Janice Evans
- Thesis title: Signal Transduction and the Events of the Egg-to-Embryo transition.

Bachelor of Science (BSc) in Biochemistry 2011
Niger Delta University

- Thesis advisors : Olu Osinowo and Chinyelu Madukosiri
- Thesis title: The effect of storage on the on the biochemical properties of young coconut water (*Cocus Nucifera*).

RESEARCH EXPERIENCE/INTERESTS

I gained introductory research experience at undergraduate level where I did a quantitative analysis on the effect of different forms of storage on the biochemical properties of young coconut water which can be used as a substitute for intra-venous fluid. My next experience was during my Master of Health Science (MHS) program, I wrote a thesis based on the events of the egg-to-embryo transition where I chronicled and expatiated the various molecules that have been implicated over time as sperm factors that trigger egg activation.

I am currently gaining hands-on experience in Dr. Philip Jordan's laboratory, where we are interested in the mechanisms involved in accurate chromosome segregation

in mammalian meiosis. My project focused on the novel role of Polo-like kinase 4 (PLK4) in regulating chromosome segregation during mammalian meiosis. Using mice as a research model, I have mastered techniques such as genotyping by PCR, germ cell extraction and chromosome spread preparations, BCA assays and western blots as well as assessing and quantifying data using the ImageJ software, Adobe Photoshop and GraphPad Prism. Though my current project focuses on the role of PLK4 in spermatogenesis, my findings have re-ignited my interest in understanding the mechanisms underlying DNA damage, its repair and genome maintenance as a whole. I look forward to the application of other cellular and biochemical mechanisms in other models to increase my understanding and fully explore DNA repair and genome integrity.

SEMINARS/EVENTS ATTENDED

- Weekly Biochemistry and Molecular Biology Seminar series
- Weekly Departmental Journal club meetings
- Monthly Cell Biology Interest group meeting
- Annual American Society of Reproductive (ASRM) meeting 2015

TEACHING EXPERIENCE

Hephzibah International Primary/Secondary School 2013-2014
Rivers State, Nigeria

- Tutoring high school students

School of Nursing, Ikot Ekpene. Akwa Ibom State, Nigeria 2011-2012

- Teaching Chemistry to pre-degree students

SCHOLARSHIP AWARD

The Nigerian Presidential Special Scholarship Scheme for Innovation and Development (PRESSID)